Univerzita Karlova

2. lékařská fakulta

Studijní program: Neurovědy

Mgr. Kristýna Kárová

Léčba míšního poranění pomocí protizánětlivých látek nebo kmenových buněk

Modifikace aktivity NFkB v léčbě míšního poranění

Anti-inflammatory compounds and stem cells in treatment of spinal cord injury

Modification of NFκB activity in different treatments of spinal cord injury

Dizertační práce

Vedoucí závěrečné práce/Školitel: Doc. Pavla Jendelová, PhD

Praha, 2018

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem řádně uvedla a citovala všechny použité prameny a literaturu. Současně prohlašuji, že práce nebyla využita k získání jiného nebo stejného titulu

Souhlasím s trvalým uložením elektronické verze mé práce v databázi systému meziuniverzitního projektu Theses.cz za účelem soustavné kontroly podobnosti kvalifikačních prací.

V Praze, 29.5.2018

Jméno KRISTÝNA KÁROVÁ

Podpis

KÁROVÁ, Kristýna. Léčba míšního poranění pomocí protizánětlivých látek nebo kmenových buněk. *[Anti-inflammatory compounds and stem cells in treatment of spinal cord injury]* Praha, 2018. 66 s., 6 příl. Dizertační práce (Ph.D.). Univerzita Karlova, 2. lékařská fakulta, Ustav experimentální medicíny. Vedoucí práce Jendelová, Pavla.

Acknowledgement

I would like to express my great appreciation to my supervisor Doc. RNDr. Pavla Jendelová, PhD. for her guidance, encouragement, constructive suggestions and critique of this research work. Her advice and support has been of great importance throughout the course of my studies. I would like to extend my thanks to the former head of Department of Neuroscience Prof. MUDr. Eva Syková, DrSc. I would also like to thank Prof. Meena Jhanwar-Uniyal and members of her team for their valuable collaboration on research projects and cytokine analyses. I wish to acknowledge the assistance and help of my colleagues, especially the help of MUDr. Lucia Machová-Urdzíková and RNDr. Jiří Růžička, PhD. I would also like to thank the technicians of the department for providing great environment and support to work smoothly. Finally, I wish to thank my family for their support during my study.

Author´s publications

Publications relevant to dissertation

Machova Urdzikova L, **Karova K***, Ruzicka J, Kloudova A, Shannon C, Dubisova J,Murali R, Kubinova S, Sykova E, Jhanwar-Uniyal M, Jendelova P. The Anti-Inflammatory Compound Curcumin Enhances Locomotor and Sensory Recovery after Spinal Cord Injury in Rats by Immunomodulation. Int J Mol Sci. 2015 Dec 31;17(1).pii: E49. doi: 10.3390/ijms17010049. **IF 3,226**

***shared first authorship**

Student´s contribution: *40% surgical procedures (50%), microscopy, immunohistochemical analysis, data analysis, statistics, article writing*

Machova Urdzikova L, Ruzicka J, **Karova K**, Kloudova A, Svobodova B, Amin A, Dubisova J, Schmidt M, Kubinova S, Jhanwar-Uniyal M, Jendelova P. A green tea polyphenol epigallocatechin-3-gallate enhances neuroregeneration after spinal cord injury by altering levels of inflammatory cytokines. Neuropharmacology. 2017 Nov;126:213-223. doi: 10.1016/j.neuropharm.2017.09.006. **IF 5,012**

Student´s contribution: 35% *surgical procedures (50%), microscopy, immunohistochemical analysis, data analysis, statistics, article writing*

Ruzicka J, Machova-Urdzikova L, Gillick J, Amemori T, Romanyuk N, **Karova K**, Zaviskova K, Dubisova J, Kubinova S, Murali R, Sykova E, Jhanwar-Uniyal M, Jendelova P. A Comparative Study of Three Different Types of Stem Cells for Treatment of Rat Spinal Cord Injury. Cell Transplant. 2017 Apr 13;26(4):585-603. doi: 10.3727/096368916X693671. **IF 3,006**

Student´s contribution: 15% *microscopy, immunohistochemical analysis, data analysis*

Urdzíková LM, Růžička J, LaBagnara M, **Kárová K**, Kubinová Š, Jiráková K, Murali R, Syková E, Jhanwar-Uniyal M, Jendelová P. Human mesenchymal stem cells modulate inflammatory cytokines after spinal cord injury in rat. Int J Mol Sci. 2014 Jun 25;15(7):11275-93. doi: 10.3390/ijms150711275 **IF 3,226**

Student´s contribution: 15% *microscopy, immunohistochemical analysis, data analysis*

Karova K, Machova-Urdzikova L, Ruzicka J, Jhanwar-Uniyal M, Jendelova P. Transplantation of neural precursors generated from spinal progenitor cells reduce inflammation in spinal cord injury via NFκB pathway modulation – *Manuscript in preparation*

Student´s contribution: 50% *surgical procedures, transplantations, microscopy, immunohistochemical analysis, data analysis, statistics, article writing*

Publications relevant to dissertation topic but not included

Ruzicka J, Urdzikova LM, Svobodova B, Amin AG, **Karova K**, Dubisova J, Zaviskova K, Kubinova S, Schmidt M, Jhanwar-Uniyal M, Jendelova P. Does combined therapy of curcumin and epigallocatechin gallate have a synergistic neuroprotective effect against spinal cord injury? Neural Regen Res. 2018 Jan;13(1):119-127. doi:10.4103/1673-5374.224379. IF **1,769**

Karova, Kristyna. Glutamate Excitotoxicity in Spinal Cord Injury. In: Rossner, Pavel et al. Nanomaterials in Biomedical Research. 1. Jendelova, Pavla. Prague. CZECH-IN, 2017, pp. 110-116. ISBN 978-80-906655-5-2.

Karova, Kristyna. Nuclear Factor - kappa B Pathway in Central Nervous System. In: Rossner, Pavel et al. Nanomaterials in Biomedical Research. 1. Jendelova, Pavla. Prague. CZECH-IN, 2017, pp. 117-123. ISBN 978-80-906655-5-2.

Publications not relevant to dissertation

Tuček L, Kočí Z, **Kárová K**, Doležalová H, Suchánek J. The Osteogenic Potential of Human Nondifferentiated and Pre-differentiated Mesenchymal Stem Cells Combined with an Osteoconductive Scaffold - Early Stage Healing. Acta Medica (Hradec Kralove). 2017;60(1):12-18. doi: 10.14712/18059694.2017.43.

Potockova H, Sinkorova J, **Karova K**, Sinkora M. The distribution of lymphoid cells in the small intestine of germ-free and conventional piglets. Dev Comp Immunol. 2015 Jul;51(1):99-107. doi: 10.1016/j.dci.2015.02.014. **IF 3,218**

Sinkora M, Stepanova K, Butler JE, Francis D, Santiago-Mateo K, Potockova H, **Karova K**, Sinkorova J. Ileal Peyer's patches are not necessary for systemic B cell development and maintenance and do not contribute significantly to the overall B cell pool in swine. J Immunol. 2011 Nov 15;187(10):5150-61. doi: 10.4049/jimmunol.1101879. **IF 4,856**

Obsah

List of abbreviations

5-HT – 5-hydroxytryptamine (serotonine)

AAT – Aspartate aminotransferase

AD – Alzheimer´s disease

ADNF – Activity dependent neurotrophic factor

ALS- Amyotrophic lateral sclerosis

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANOVA – Analysis of variance

AP-1 – Activator protein 1

Arg-1 – Arginase 1

ATP – Adenosine triphosphate

AQP4 – Aquaporine 4

BBB – Blood-brain barrier

BBB score – Basso Beattie Bresnahan open field test

Bad – Bcl-2-associated death promoter protein

Bax – Bcl-2-associated X protein

Bak – Bcl-2 homologous antagonist/killer

Bcl-2 – B-cell lymphoma 2 protein

Bcl-$_{XL}$ – B-cell lymphoma-extra large

BCR – B cell receptor

BDNF – Brain derived neurotrophic factor

bFGF – Basic fibroblast growth factor

BSCB – Blood-spinal cord barrier

Cap 23 (Basp1) - brain abundant membrane attached signal protein 1

Casp 3 – Caspase 3

CCL-2 - C-C motif chemokine ligand 2

CCL20 - C-C motif chemokine ligand 20

CD - Cluster of differentiation

CFDA-SE - Carboxyfluorescein diacetate succinimidyl ester

ChAT – Choline acetyltransferase

c-Myc – c-*myc*proto-oncogene

CNS – Central nervous system

Cntf – Ciliary neurotrophic factor

$\cdot CO_3$ - Carbonate radical

Cox-2 – Cyclooxygenase 2

CSPGs – Chondroitin sulphate proteoglycans

CST – Corticospinal tract

DAB – 3´-diaminobenzidine

DAMPs – Danger associated molecular patterns

DCN – Dorsal column nuclei

DCX – Doublecortin

DG – Dentate gyrus

DMEM/F12 - Dulbecco's modified eagle medium/nutrient mixture F-12

DNA – Deoxyribonucleic acid

dsRNA – Double stranded RNA

EGCG - Epigallocatechin-3-gallate

EGF – Epidermal growth factor

ER – Endoplasmic reticulum

ESC – Embryonic stem cell

FACS – Fluorescence-activated cell sorting

FASL – Fas ligand (CD95L)

FBS – Fetal bovine serum

FGF – Fibroblast growth factor

GABA – Gamma-aminobutyric acid

GAP43 – Growth associated protein 43

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GFAP – Glial fibrillary acidic protein

GFP – Green fluorescence protein

GDF-3 - Growth differentiation factor-3

GDH1 – Glutamate dehydrogenase 1

GDH2 – Glutamate dehydrogenase 2

GDNF – Glial cell-derived neurotrophic factor

GLAST (EAAT-1) – Glutamate aspartate transporter (Excitatory amino acid transporter 1)

GLT-1 (EAAT-2) – Glutamate transporter 1 (Excitatory amino acid transporter 2)

GS – Glutamate synthetase

H_2O_2 - Hydroxide peroxide

HD – Huntington´s disease

HGF – Hepatocyte growth factor

HMGB1 - High-mobility group protein (B)1

HSP – Heat shock protein

HT22 – Mouse hippocampal neuronal cell line

IAPs – Inhibitors of apoptosis

IFNγ – Interferon γ

IgG – Immunoglobuline G

IHC - Immunohistochemistry

IL – Interleukin

iNOS – Inducible nitrous oxide synthase

IκB - Inhibitor of kappa B

IKK(α, β) – Inhibitor κB kinase (α, B)

IKKγ (NEMO) - Inhibitor κB kinase γ (NF-kappa-B essential modulator)

IP3 – Inositol-3-phosphate

iPS-NP – Induced pluripotent stem cells - derived neural precursors

Irf5 - Interferon regulatory factor 5

JAK2 – Janus kinase 2

KLF4 - Kruppel-like factor 7

KLF7 - Kruppel-like factor 7

Ku80 – protein complex involved in DNA repair, named by patient in whom discovery was made

LIF – Leukemia inhibitory factor

LIN 28 – RNA binding protein, pluripotency factor

LLOQ – Lower limit of quantification

Lox - Lipoxygenase

L-myc – Proto-oncogene

LPS – Lipopolysaccharide

MAP-2 – Microtubule associated protein 2

MBP – Myelin basic protein

MCP-1 - Monocyte attracting protein-1

MDA – Malondialdehyde

MEM – Minimal essential medium

MFI – Mean fluorescence intensity

MHCI – Major histocompatibility protein class I

MIP1α – Macrophage inflammatory protein 1α

Mn-SOD – Manganese superoxide dismutase

MMP-9 – Matrix metalloproteinase - 9

MPO – Myeloperoxidase

mPTP (MPT) - Mitochondrial permeability transition pores

Mrc1 - Mannose receptor C type 1

MS – Multiple sclerosis

MSC – Mesenchymal stem cells

MTCO2 - mitochondrially encoded cytochrome c oxidase II

mTOR – Mammalian target of rapamycine

NANOG – Transcription factor maintaining pluripotency, named after Celtic legend Tir na nOg

NeuN - Neuronal nuclear antigen

NF200 – Neurofilament 200 kDa

NFκB – Nuclear factor kappa B

$NG2^+$ - Neuron-glial antigen 2^+

NGF – Neural growth factor

NLS – Nuclear location signal

NPCs – Neural precursor cells

NSCs – Neural stem cells

NMDA – N-methyl-D-aspartate

NO – Nitric oxide

·NO - Nitric oxide radical

Nogo-A - Neurite outgrowth inhibitor A

NOS – Nitrous oxide synthase

NT3 – Neurotrophin 3

$O^{\cdot -}$ - Superoxide anion

Oct ¾ - Octamer-binding transcription factor ¾

Olig1 – Oligodendrocyte transcription factor 1

Olig2 - Oligodendrocyte transcription factor 2

$P2X_n/P2Y_n$ – Ionotropic and G-protein coupled ATP receptors

p65 – Protein 65 kDa

PAG – Phosphate activated glutaminase

PAMPs – Pathogen associated molecular patterns

PBS – Phosphate buffered saline

PHA – Paraformaldehyde

PD – Parkinson´s disease

PDGF – Platelet derived growth factor

PECAM-1 – Platelet endothelial cell adhesion molecule 1

PEDF – Pigment epithelium-derived factor

PRRs – Pattern recognition receptors

RAGE - Receptor for advanced glycation endproducts

RAGs – Regeneration associated proteins

RANTES/CCL5 - Regulated on activation, normal T cell expressed and secreted

REX-1/zfp42 - Zinc finger protein 42

RHD – Rel homology domain

RM – Repeated measurement

RNA – Ribonucleic acid

ROS – Reactive oxygen species

RPE – R-phycoerythrin

SCF – Stem cell factor

SCI – Spinal cord injury

SEM – Standard error of the mean

shRNA – Short hairpin RNA

SN1 – System N1

SNAT - Sodium-coupled neutral amino acid

SNK test – Student, Neumann, Keuls test

SOD – Superoxide dismutase

SOX2 – (sex determining region Y) – box 2

SOX9 – SRY-box 9

SOX11 - SRY-related HMG-box 11

SPC-01 – Spinal precursor cell line 01

STAT - Signal transducer and activator of transcription

SVZ – Subventricular zone

TAD – Transactivation domain

T_c – Cytotoxic T lymphocyte

TCR – T cell receptor

TGFβ – Transforming growth factor β

T_h1 – Helper T lymphocyte type 1

T_h2 - Helper T lymphocyte type 2

T_h17 – Helper T lymphocyte type 17

TNFα – Tumor necrosis factor α

TNFR – Tumor necrosis factor receptor

TLRs – Toll-like receptors

TLR2 – Toll-like receptor 2

TLR4 – Toll-like receptor 4

Tuj1 – Neuron specific class III β tubulin

VEGF – Vascular endothelial growth factor

VSV-G – Vesicular stromatitis virus glycoprotein

Abstrakt

Navzdory intenzivnímu vědeckému úsilí zůstává poranění míchy těžkým neurologickým stavem, které nemá žádnou léčbu. V současné době je terapie založena na zmírnění tlaku chirurgickou dekompresí míchy, podávání methylprednisolonu a fyzioterapie. Tato práce se zabývá terapeutickými účinky protizánětlivých sloučenin a tří typů kmenových buněk v modelu balónkové kompresní léze míchy u potkanů. Přírodní sloučeniny epigallocatechin-3-galát (EGCG) nebo kurkumin jsme aplikovali *in situ* a poté intraperitoneálně každý den až po dobu 28 dnů. Lidské mesenchymální kmenové buňky z kostní dřeně (MSC), lidské spinální neurální prekurzory (SPC-01) a neurální prekurzory derivované z lidských indukovaných pluripotentních kmenových buněk (iPS-NP) jsme transplantovali intratekálně (MSCs) nebo pomocí spinální injekce potkanům 7 dní po indukci míšního poranění. Změny motoriky a citlivosti zadních končetin jsme hodnotili behaviorálními testy. Sledovali jsme rovněž míru zachování šedé/bílé hmoty a velikost kavity léze. Přežití a diferenciaci transplantovaných buněk, aktivaci klasické dráhy NFκB, gliovou jizvu (GFAP+) a růst axonálních kolaterál (GAP43+) jsme studovali pomocí imunohistochemie. Pro stanovení hladin sekretovaných cytokinů a chemokinů (MIP1α, IL-4, IL-1β, IL-2, IL-6, IL-12p70, TNFα, RANTES) jsme použili analýzu Luminex. Hodnotili jsme i změny transkripce endogenních genů souvisejících s regenerací tkáně v prvním měsíci léčby. Zjistili jsme, že po míšním poranění dochází ke zvýšení aktivity NFκB ve dvou vlnách a to 3 a 28 dní po poranění, které doprovází zvýšení zánětlivých cytokinů. Obě protizánětlivé sloučeniny silně potlačily aktivaci NFkB (p65), zatímco buněčná terapie (SPC-01, MSC) inhibovala druhou vlnu aktivity 28 dní po poranění. Všechny testované způsoby léčby vedly ke zlepšení motorických funkcí, nejlepší výsledky byly pozorované u zvířat transplantovaných neurálními prekurzory z iPS. EGCG i kurkumin snížily produkci chemokinů MIP1α a RANTES, což bylo pozorováno i po aplikaci obou typů neurálních prekurzorů. Všechny léčebné přístupy snížily produkci prozánětlivých TNFα, IL-2 a IL-1β s výjimkou EGCG, kde došlo k počátečnímu nárůstu těchto proteinů a jejich postupnému snižování. Neuroprotektivní IL-4 byl významně zvýšen po léčbě EGCG a SPC-01. Zvýšená produkce IL-6 a IL-12p70 u zvířat léčených kurkuminem a oběma typy neurálních prekurzorů korelovala s výrazným růstem axonálních kolaterál a funkčním zotavením, ale nikoliv s klasickou dráhou NFκB. To naznačuje, že tyto cytokiny jsou regulovány jinými transkripčními faktory aktivovanými míšním

poraněním. Všechny terapie s výjimkou EGCG vedly ke snížení astrogliózy. Naše výsledky ukazují na silné imunomodulační účinky všech testovaných terapií, které vedou ke zlepšení pohybových schopností léčených potkanů a k regeneraci míšní tkáně po poranění.

Klíčová slova

Míšní poranění, zánět centrálního nervového sytému, kanonická dráha NFκB (p65), imunomodulace, EGCG, kurkumin, terapie kmenovými buňkami, neuroregenerace

Abstract

Despite intense scientific efforts, spinal cord injury (SCI) remains to be a severe neurological condition that has no treatment. Currently, therapy is based on alleviating pressure by surgical spinal cord decompression, administration of methylprednisolone and physical therapy. In this study, therapeutic effects of anti-inflammatory compounds and of three types of stem cells were tested in a balloon compression model of spinal cord injury in rats. Natural compounds epigallocatechin-3-gallate (EGCG) or curcumin were administered *in situ* and then intraperitoneally every day for up to 28 days. Human bone marrow mesenchymal stem cells (MSCs), human spinal neural precursors (SPC-01) and neural precursors derived from human induced pluripotent stem cells (iPS-NPs) were transplanted intrathecally (MSCs) or via spinal injection into immunosuppressed rats 7 days after induction of SCI. To determine effects of therapies, changes in motor function was tested by open field test BBB, flat beam test and score, Plantar test and rotarod. Morphometric analysis was used to assess gray/matter sparing and cavity size. Immunohistochemistry was used to determine survival and differentiation of transplanted cells, activation of classical pathway of NFκB (p65 nuclear translocation), astroglial activation (GFAP) and axonal sprouting (GAP43). To determine levels of produced cytokines and chemokines (MIP1α, IL-4, IL-1β, IL-2, IL-6, IL-12p70, TNFα, RANTES), Luminex custom 8-plex assay was used. After SCI, there are two spikes in NFκB activity in the first month, 3 and 28 days after injury induction. Both anti-inflammatory compounds strongly inhibited NFκB (p65) activation, while MSCs and SPC-01 prevented the second spike of activity. All tested treatments resulted in improved motor function, with the best outcomes observed in animals transplanted with iPS-NPs. Both EGCG and curcumin lowered production of chemokines MIP1α and RANTES, which was also observed after treatment with NPs. All treatments caused downregulation of pro-inflammatory TNFα, IL-2, IL-1β except for EGCG, where initial increase occurred and later subsided. Neuroprotective IL-4 was significantly higher after treatment with EGCG and SPC-01. We found strong upregulation of IL-6 and IL-12p70 in animals treated with curcumin and either type of neural precursors, which correlated with strong enhancement of axonal sprouting and functional recovery, but not with classical NFκB pathway suggesting that these cytokines are regulated by different transcription factor in SCI. All treatments except for EGCG resulted in lower astrogliosis. Our results demonstrate strong immunomodulatory effects of all treatments

resulting in improved tissue preservation and regeneration and locomotor skills of treated rats.

Keywords

Spinal cord injury, neuroinflammation, NFκB (p65) canonical pathway, immunomodulation, EGCG, curcumin, stem cell therapy, neuroregeneration

1. Introduction

Spinal cord injury is a severe condition for which an effective treatment is still not available. After the initial irreversible mechanical damage that results in necrosis and destruction of neural connections, several secondary processes take place and can last for several months until lesion cavity is established. Location and severity of spinal cord lesion are used to classify injury outcome using ASIA impairment scale (Tab. 1). Current research efforts are focused on these processes, which are believed to be, to certain extend, reversible or it is possible to modulate them. Such modulation would be beneficial to the patient by preventing further degenerative damage to the spared tissue, which is known to progress over several months after the injury itself. Secondary processes include edema, disruption of the blood-spinal cord barrier (BSCB), ischemia, inflammation, oxidative stress, glutamate excitotoxicity, and apoptosis which all take place immediately after the mechanical insult. Molecular cascades in these processes cross talk and progress by positive feedback loops or by employment of various molecular and cellular elements at different time points. This is particularly relevant in the inflammatory response.

ASIA Impairment Scale (AIS)

A = Complete. No sensory or motor function is preserved in the sacral segments S4-5.

B = Sensory Incomplete. Sensory but not motor function is preserved below the neurological level and includes the sacral segments S4-5 (light touch or pin prick at S4-5 or deep anal pressure) AND no motor function is preserved more than three levels below the motor level on either side of the body.

C = Motor Incomplete. Motor function is preserved at the most caudal sacral segments for voluntary anal contraction (VAC) OR the patient meets the criteria for sensory incomplete status (sensory function preserved at the most caudal sacral segments (S4-S5) by LT, PP or DAP), and has some sparing of motor function more than three levels below the ipsilateral motor level on either side of the body.
(This includes key or non-key muscle functions to determine motor incomplete status.) For AIS C – less than half of key muscle functions below the single NLI have a muscle grade ≥ 3.

D = Motor Incomplete. Motor incomplete status as defined above, with at least half (half or more) of key muscle functions below the single NLI having a muscle grade ≥ 3.

E = Normal. If sensation and motor function as tested with the ISNCSCI are graded as normal in all segments, and the patient had prior deficits, then the AIS grade is E. Someone without an initial SCI does not receive an AIS grade.

Using ND: To document the sensory, motor and NLI levels, the ASIA Impairment Scale grade, and/or the zone of partial preservation (ZPP) when they are unable to be determined based on the examination results.

Tab. 1 Asia impairment scale (http://www.oxfordmedicaleducation.com)

1.1. Edema and ischemia

Edema occurs as a result of ion imbalance and increased membrane permeability, where there is influx of Na^+ and Ca^{2+} ions into the cells, which is coupled with water molecules influx (Ahmed et al., 2017). Water enters cells via channels called aquaporines. Aquaporine 4 (AQP4) is the most abundant out of the 7 identified subtypes in the rodent brain and is mainly expressed in astrocytic endfeet in contact with blood vessels (Sun et al., 2017), (Badaut et al., 2011), its expression after SCI is upregulated and correlates with the extent of SCI edema (Li et al., 2014) (Fig.1). Overexpression of AQP4 is associated with increased adhesion facilitating glial scar formation (Li et al., 2014). This ultimately results in swelling of cells which can lead to their bursting, promoting more inflammation and oxidative stress.

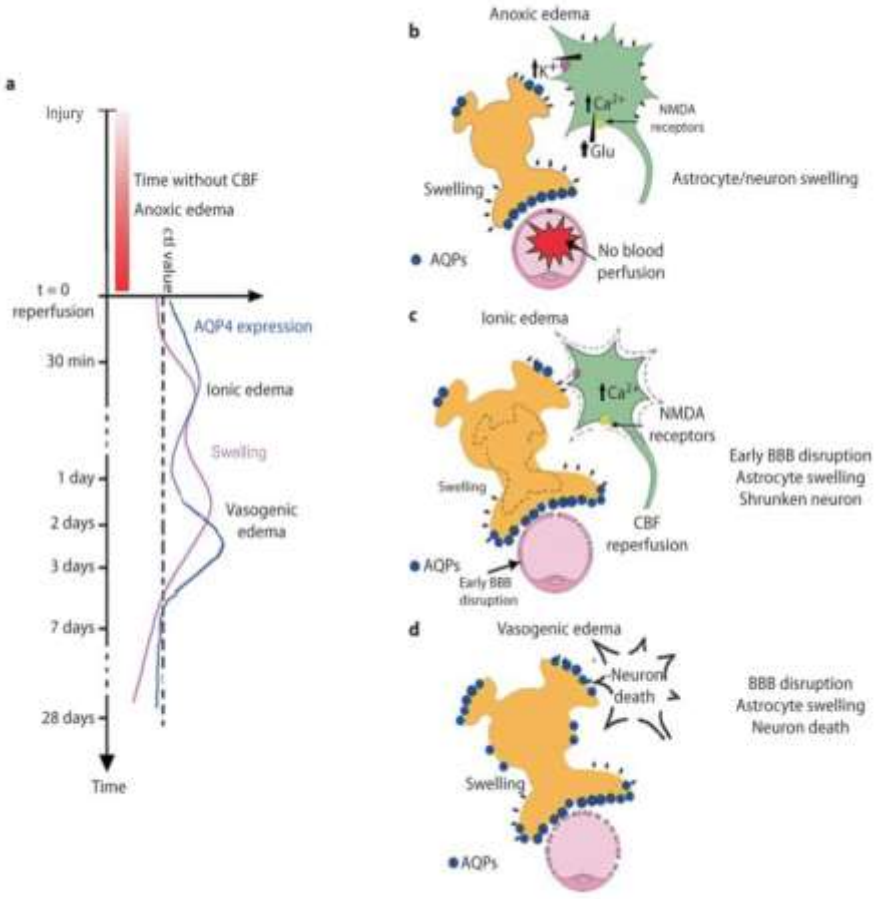

Fig. 1 Timeline and description of different types of edema, caused by ion imbalance, blood-brain barrier (BBB) disruption and AQPs upregulation resulting in shrinking of neurons and their subsequent death (Badaut et al., 2011).

Alleviating pressure created by swollen tissue is one of the measures surgeons take on patients with spinal cord injury. Surgical decompression can create more space so that swelling can subside destroying less of the healthy surrounding tissue. However, due to variability in timing and severity of injury, benefits of early (less than 24 hours) decompression have been deemed inconclusive. This approach is suggested to be presented to patients as an option to improve outcome (Fehlings et al., 2017).

1.2. Excitotoxicity

In pathological states like spinal cord injury, glutamate is released from dying necrotic cells and its extracellular concentration rises sharply. Glutamate (glutamic acid) is the most abundant neurotransmitter in the central nervous system and functions as excitatory mediator to trigger depolarizations of postsynaptic neurons, its levels in the brain can locally reach up to 10^4 μM. To ensure high fidelity of transmission, its extracellular levels are strictly controlled and kept at less than 1-2 μM by reuptake mechanism provided by adjacent astrocytes (Julio-Pieper et al., 2011). Two main glutamate transporters, GLAST (EAAT-1) and GLT-1 (EAAT-2) are highly expressed in astrocytic membranes with GLT-1 making up 80-90% (Bajrektarevic and Nistri, 2017; Hertz and Rothman, 2017). Astrocytes are closely associated with neurons not only to provide metabolic support and mediate connection to blood stream, but also to deplete released glutamate, thanks to their participation in synaptic transmission. Astrocytes express glutamate receptors (most important is metabotropic glutamate receptor 5, mGluR5) that detect synaptic activity and help regulate signal transmission and neuronal excitability, making them an important component of the so called tripartite synapse. Apart from astrocytic response to transmission via mGluR5 (Panatier and Robitaille, 2016), subsequent Ca^{2+} dependent cellular activity and release of glial proteins known as gliotransmitters, astrocytes actively transport excess of glutamate from the synapse to prevent prolonged, toxic stimulation. This transport is coupled with Na^+ uptake, which is dependent on gradients generated by Na^+/K^+ ATPase (Wang et al., 2005; Rose et al., 2009; Yaster et al., 2011). It has been reported that large energy demand for this transport during high pathological activity in brain ischemia impairs the Na^+/K^+ ATPase function due to ATP consumption, reversing glutamate transport outwards and causing more damage (Phillis JW, 2000).

Glutamate transported into astrocytes is converted to glutamine by glutamine synthetase (GS) (Bajrektarevic and Nistri, 2017; Hertz and Rothman, 2017). Astrocytes

compensate for the missing glutamate by synthetizing new molecules from α – ketoglutarate catalyzed by aspartate aminotransferase (AAT) or glutamate dehydrogenase, which has two forms GHD1 and GHD2 (McKenna et al., 2016; Hertz and Rothman, 2017). Neurons do not express glutamine synthetase or pyruvate carboxylase and depend on *de novo* synthesis of glutamate from astrocytes, which are also the source of glutamine. It is evident, that conversion of glutamate to non-excitatory amino acid glutamine is energy costly but essential to prevent glutamate excitotoxicity, homeostasis of CNS environment and correct transmission. Astrocytes release glutamine into extracellular space via SN1 transporters and glutamine is then taken up by neurons predominantly by members of sodium-coupled SNAT transporter family, which are mostly located in the bodies and dendrites suggesting that there may be another unidentified transporter in axonal terminals (Kanamori and Ross, 2006; Hagglund et al., 2015). Glutamine is converted into glutamate in neurons by phosphate activated glutaminase (PAG) to replenish neuronal glutamate levels. It has been shown that this complex cycle of glutamate and glutamine between astrocytes and neurons that provide energy and safe and stable handling of glutamate is impaired after spinal cord injury. This impairment is characterized by decreased activity of glutamate synthetase and phosphate activated glutaminase, as well as increased activity of glutamate decarboxylase, enzyme catalyzing glutamate conversion into gamma-aminobutyric acid (GABA) inhibitory neurotransmitter (Diaz-Ruiz et al., 2016).

Due to the mechanical insult to neural tissue, not only neurons are damaged but also the supporting glia, which results in compromised glutamate uptake by astrocytes. This causes prolonged presence of large amounts of glutamate binding to N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. Constant activation of glutamate receptors results in more glutamate being released by spared neurons into the synaptic clefts of neurons further from the injury site causing their overstimulation as well. Neurons experience persisting influx of Ca^{2+} ions which exceeds the cell´s buffering ability and activate many pathways, which becomes metabolically exhausting. Moreover, excess Ca^{2+} is taken up by mitochondria and triggers release of cytochrome c, leading to intrinsically activated cell death. Cell death activated internally is a response to various imbalances, such as imbalance of the endoplasmic reticulum (ER), mitochondrial function, low levels of growth factors or anti-apoptotic factors, radiation, oxidative stress or hypoxia (Elmore, 2007). Mitochondria release cytochrome c trough mitochondrial permeability transition pores

(mPTP or MPT), which open due to Ca^{2+} accumulation (Orrenius et al., 2003; Elmore, 2007). Cytochrome c then binds to inositol-3-phosphate (IP3) on endoplasmic reticulum and potentiates Ca^{2+} release, increasing its intracellular levels even more. It is well established, that apoptosis is also regulated by pro-apoptotic (i. e. Bax, Bak, Bad) and anti-apoptotic (i. e. Bcl-2, Bcl- $_{XL}$) proteins associated with mitochondrial membrane (Birkinshaw and Czabotar, 2017) (D'Orsi et al., 2017). Anti-apoptotic proteins reduce release of Ca^{2+} from ER, alter permeability of mitochondria to influx of Ca^{2+} and the release of cytochrome c. Pro-apoptotic proteins have been shown to have opposite effect (D'Orsi et al., 2017). Calcium levels are strictly regulated to maintain low intracellular concentration, with its transient increases to put proper cellular responses in motion. However, insufficiently buffered levels of calcium are a signal to trigger apoptosis. Glutamate excitotoxicity is also closely connected with another detrimental as well as beneficial process, inflammation. It has been shown, that glutamic acid activates the NFκB pathway, which is a crucial regulator cytokine production, production of chemokines, adhesive molecules and pro- and anti-apoptotic proteins.

1.3. Oxidative stress and inflammation

Free radicals produced by cellular metabolism are released from cells upon spinal cord injury. Molecules containing unpaired electron are highly reactive and disrupt surrounding structures. Free radicals are associated with oxygen and cause lipid peroxidation, which occurs as early as 5 mins after injury (Garcia et al., 2016). Notoriously studied are reactive oxygen (ROS) or nitrous species (NOS), which include superoxide anion $O^{\bullet -}$, hydroxide peroxide H_2O_2, hydroxyl radical $^{\bullet}OH$, carbonate radical $^{\bullet}CO_3$, nitric oxide radical $NO^{\bullet}$, which is known to be primary product of NO synthase (Martin, 2009; Hall et al., 2016). Under physiological circumstances they are buffered by a cascade of reactions catalyzed by several enzymes like superoxide dismutase (SOD), catalase or glutathione peroxidase (Zabel et al., 2017). After injury, this buffering ability is compromised and concentrations of radicals exceed the antioxidant capacity, which leads to lipid peroxidation and disruption of cellular membranes. This is a significant contributor to secondary damage like demyelination in tissue so rich in lipids.

Inflammation is one of the most complex processes taking place after spinal cord injury. It is mediated by a number of cytokines and specialized cells resident in the central nervous system or cells that migrate into the lesion area from blood. The earliest response can be anticipated from resident immune cells like microglia, but also from astrocytes that are known to be activated and secrete a battery of proteins modulating the immune response. Cells within the injured tissue release a number of Danger Associated Molecular Patterns signaling damage and attracting immune cells towards the site. Microglia is the immunocompetent cell type of the CNS, displaying a ramified morphology under physiological condition. Their role in healthy brain and spinal cord is to survey their surroundings, screening for any pathogens or changes in homeostasis (Kettenmann et al., 2011). Quickly after detecting the injury, microglia dramatically change their shape and become amoeboid with increased phagocytic ability (Kettenmann et al., 2011). Various chemoattractant molecules, chemokines, are released in the injured tissue attracting immune cells to move towards the injury. Chemokines like C-C motif chemokine ligand 2 (CCL2), Macrophage inflammatory protein 1α, (MIP1α), Regulated on activation, normal T cell expressed and secreted (RANTES) facilitate infiltration of cells to clear out debris and to produce a number of cytokines and other proteins to promote inflammation and attract immune cells from periphery, e.g. neutrophils, which also phagocytose and release cytokines, ROS, matrix metalloproteinase-9 (MMP-9) and myeloperoxidase (MPO) both implicated in BSCB disruption (Ahmed et al., 2017). Activation of microglia has been shown to precede astrogliosis characterized by increased expression of glial fibrillary acidic protein (GFAP), but also by astrocytic production of a variety of cytokines like Tumor necrosis factor α (TNF-α), interleukines IL-1β, IL-6 and chemokines (Tian et al., 2007) (Liddelow et al., 2017) (Bellaver et al., 2017). Classically activated (via ligands of Toll-like receptors (TLRs) - lipopolysaccharide (LPS), interferon γ (IFNγ), monocyte attracting protein-1 (MCP-1)) (Ahmed et al., 2017) (Kong and Gao, 2017) microglia and infiltrated macrophages (M1 phenotype), expressing CD86 and Irf5, produce pro-inflammatory cytokines like IL-1β, IL-6, TNFα, IL-12, IL-15, IL-18, but also anti-inflammatory cytokines like IL-10, transforming growth factor β (TGFβ). They also promote oxidative stress via release of reactive oxygen/nitrogen species and induction of inducible nitrous oxide synthase (iNOS) production (Kong and Gao, 2017; Marcol et al., 2017) (Fig. 2).

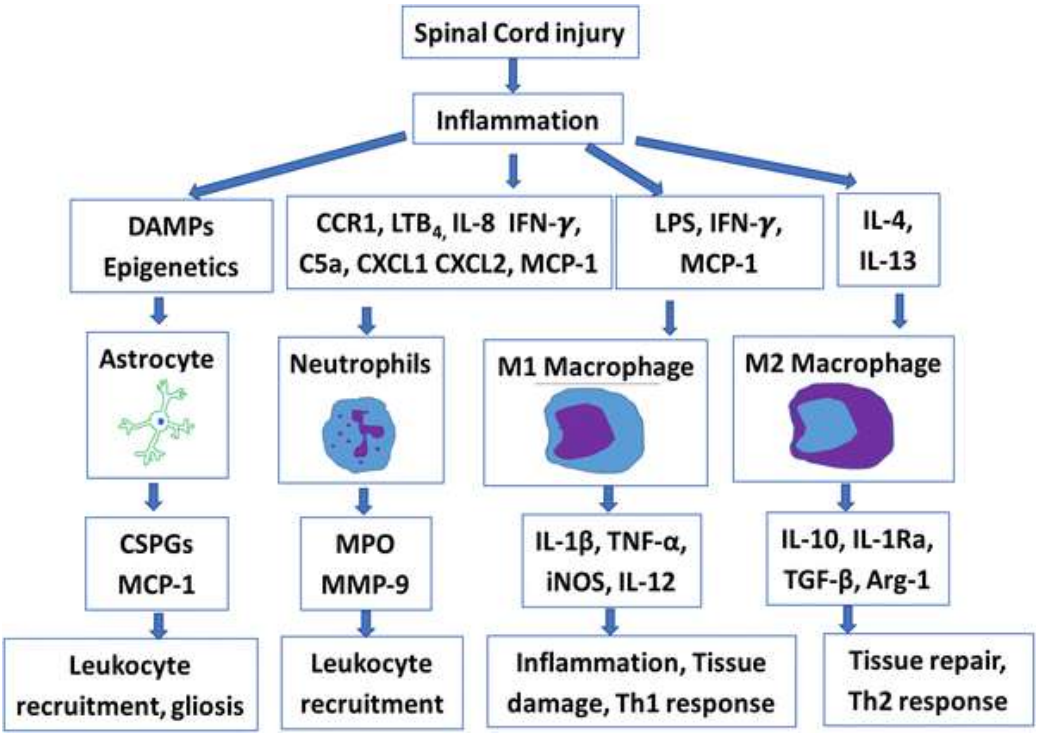

Fig.2 Inflammatory response after spinal cord injury (Ahmed et al., 2017).

This M1 type of macrophages is present in the spinal cord lesion and adjacent areas immediately as a result of microglia transformation and later from the periphery and their levels are maintained (Chen et al., 2015; Wang et al., 2015). Alternatively activated microglia or monocyte derived macrophages M2 ($CD163^+$, $Mrc1^+$, $Arg\text{-}1^+$) appear to be transiently induced (Chen et al., 2015) by interleukines IL-4, IL-10, IL-13 and their activity is believed to be neuroprotective. It has been shown that adoptive transfer of M2 macrophages resulted in improved functional outcome (Ma et al., 2015) by stimulating healing and reducing levels of inflammation. These cells can produce anti-inflammatory IL-10, interleukine 1 receptor antagonist (IL-1ra) or healing promoting TGFβ (Ahmed et al., 2017), (Kong and Gao, 2017; Zhao et al., 2017). However, clearly distinguishing M1/M2 phenotypes and interpretations of their actions in vivo is challenging. T-lymphocytes have been detected in rodents as early as 7 days after injury, peaking at around 42 days after SCI. Several subtypes of T-lymphocytes are at play, most studied are the helper T lymphocyte subsets Th1, Th2, Th17 expressing CD4 and cytotoxic lymphocytes (T_c) expressing CD8 (Garcia et al., 2016). Cytotoxic T lymphocytes rapidly migrate into the spinal cord lesion and persist for 2 weeks, with dramatic subsequent decline (Wu et al., 2017). Th1 lymphocytes produce IFNγ acting on macrophages and polarizing them towards M1 phenotype (Hu et al.,

2016). In contrast, Th2 lymphocytes produce IL-4, IL-10, IL-13 associated with anti-inflammatory response and M2 polarization. Th2 have also been found to produce growth factors neural growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophins NT3, 4 and 5, which are all neuroprotective (Garcia et al., 2016; Hu et al., 2016). Th17 are attracted to injury site by CCL20 and produce IL-17, which enhances IL-6 and IL-8 production deepening the immune reaction (Garcia et al., 2016; Hu et al., 2016).

1.3.1. NFκB pathway

The family of transcription factors NFκB is ubiquitously present in all cell types and is involved in many processes throughout the life of a cell. Those include immunological responses, inflammation, cell survival, growth and development. In the central nervous system, NFκB activity is also involved in regulation of learning and memory (Zhang et al., 2012; Shih et al., 2015). There are 5 members of the NFκB family, RelA (p65), RelB, c-Rel, NFκB1, NFκB2, which form a variety of dimers that bind to κB enhancers to trigger transcription of number of genes (SC, 2010). They all contain the Rel Homology Domain (RHD) providing the ability for DNA binding and dimerization. Only RelA, RelB and c-Rel, however, contain the transactivation domain (TAD) necessary for transcribing appropriate genes by NFκB dimers (Wan and Lenardo, 2009). Up to 500 genes transcribed by this pathway have been identified. These include *Cox-2, Lox, iNOS, IL-1, IL-6, IL-8, TNFα*, various chemokines like MIP-1α, RANTES (CCL5), etc. (Shih et al., 2015; Christian et al., 2016). This is fine - tuned by a number of possible modifications not only of Rel proteins, but also of other participants in the machinery, including Inhibitor of kappa B (IκB) family members, which inhibit NFκB dimers in the cytoplasm and Rel – associating proteins. Most common modifications are phosphorylation, ubiquitination, acetylation, sumoylation or nitrosylation, which predominantly act to enhance the deoxyribonucleic acid (DNA) binding activity (Wan and Lenardo, 2009). Correct stimulus leading up to specific gene expression is also regulated by specific chromatin modifications (Wan and Lenardo, 2009). Evidently, this pathway is essential for defense against pathogens and for regulation of apoptosis and cell survival of stressed and injured cells, which is achieved by production of a number of pro- and anti – apoptotic proteins, chemokines, adhesion molecules, NFκB proteins, matrix metalloproteinases, pro- and anti – inflammatory cytokines and more. Cytokines produced by somatic or immune cells like TNF-α or IL-1β are also known to trigger

NFκB pathway. Two NFκB pathways have been discriminated, canonical and non-canonical, and it has been demonstrated that activation of canonical pathway can suppress the basal activity of non – canonical pathway (Gray et al., 2014).

NFκB pathway is predominantly activated by various stimuli related to microbial or viral infiltration and/or tissue injury (mechanical, UV light). PAMPs, Pathogen Associated Molecular Patterns and DAMPs, Danger Associated Molecular Patterns are generally recognized by the so called PRRs – Pattern Recognition Receptors. PAMPs are components of bacterial bodies or viral particles, such as lipoproteins, glycoproteins, lipopolysaccharide (LPS), double-stranded RNA (dsRNA), un-methylated CpG DNA motifs, etc. DAMPs on the other hand are molecules released by injured or stressed cells like heat shock proteins (HSP), high mobility group box protein 1 (HMGB1) ATP, S100s, uric acid (Tang et al., 2012). A variety of PRRs developed on cellular surface and in cytosol. Typically, toll-like receptors are anchored in the cellular membrane and are able to bind PAMPs and DAMPs, triggering a specific cascade to relay appropriate response. Examples of DAMPs and their PRRs are RAGE – S100s and TLR4 – HGSP or HMGB1, ATP - $P2X_n/P2Y_n$ (Tang et al., 2012; Kierdorf and Prinz, 2013).

The most pronounced and established responses in form of NFκB activation are seen in immune cells present throughout the body, which in the CNS are microglia. Immunity however is not the only system relying on this pathway. As mentioned, it facilitates many other processes and has been documented to be an important regulator of neurological functions.

Canonical pathway is the classical NFκB pathway which is activated by ligands binding to a variety of TLRs, TNFR, IL-1R, TCR, BCR, CD30 and CD40, which allows for recruitment of multiple adaptor proteins with kinase activity such as MEKK1, RIP, CIKS (Min-Jean Yin, 1988; Prajapati and Gaynor, 2002) activating inhibitor κB kinase (IKK) complex by phosphorylation. This complex consists of IKKα, IKKβ and regulatory IKKγ (NEMO) subunits all essential in canonical NFκB pathway (Wan and Lenardo, 2009). Interestingly, IKK complex has the ability of auto - phosphorylation at specific serine cluster, which ensures a decrease in IKK activity and is one of the mechanisms preventing prolonged activity (Mireille Delhase, 1999; Prajapati and Gaynor, 2002). NFκB proteins form various combinations of dimers in the cytoplasm, the most common dimers in canonical pathway contain RelA or c-Rel (Lawrence, 2009). Composition of NFκB dimers is tissue or cell type specific and distinguishes their function, response and whether they are protective or not. The most common

p50/RelA (p65) dimer has been widely studied and has been determined to be crucial in immune response to pathogens and injury, but its prolonged aberrant activity is implicated in a number of chronic inflammatory diseases, neurodegenerative diseases (MS, ALS, AD, PD, HD), schizophrenia, autoimmune diseases, malignancies (Roussos et al., 2013) (Pizzi et al., 2009) (Yuste et al., 2015) (Lawrence, 2009; Shih et al., 2015). NFκB dimers are blocked from nuclear translocation by IκB molecules which mask their nuclear location signal (NLS) in all unstimulated nucleated cells. NFκB classical pathway is independent of *de novo* protein synthesis and a crucial step is the release from its inhibitory complex (Wan and Lenardo, 2009; SC, 2010; Shih et al., 2015). There are 8 members of IκB molecules, genes of some (IκBα, IκBβ and IκBε) are targets of NFκB transcription pathway, which creates a negative feedback loop (Wan and Lenardo, 2009). After stimulation and activation of IKK complex, these IκB inhibitors are phosphorylated and dissociate from NFκB dimers. Ultimately, phosphorylated IκB are ubiquitinated and degraded in proteasome (Wan and Lenardo, 2009; SC, 2010; Shih et al., 2015). When NFκB dimer is free, it translocates into the nucleus and binds to κB enhancers to initiate transcription of appropriate genes (Fig. 3).

NFκB in astrocytes

Astrocytes are essential for CNS activity, it is well established, that astrocytes are activated in pathological conditions like infection or trauma. They form a physical barrier around the lesion and help facilitate wound healing and are in fact necessary for axonal regrowth (Pekny et al., 2014; Anderson et al., 2016). However, several factors, including the glial scar, prevent regeneration by creating inhibitory environment, which contributes to a very limited intrinsic regeneration ability of CNS tissue, i.e. presence of inhibiting factors and absence of growth stimulating ones (Anderson et al., 2016) (Cregg et al., 2014).

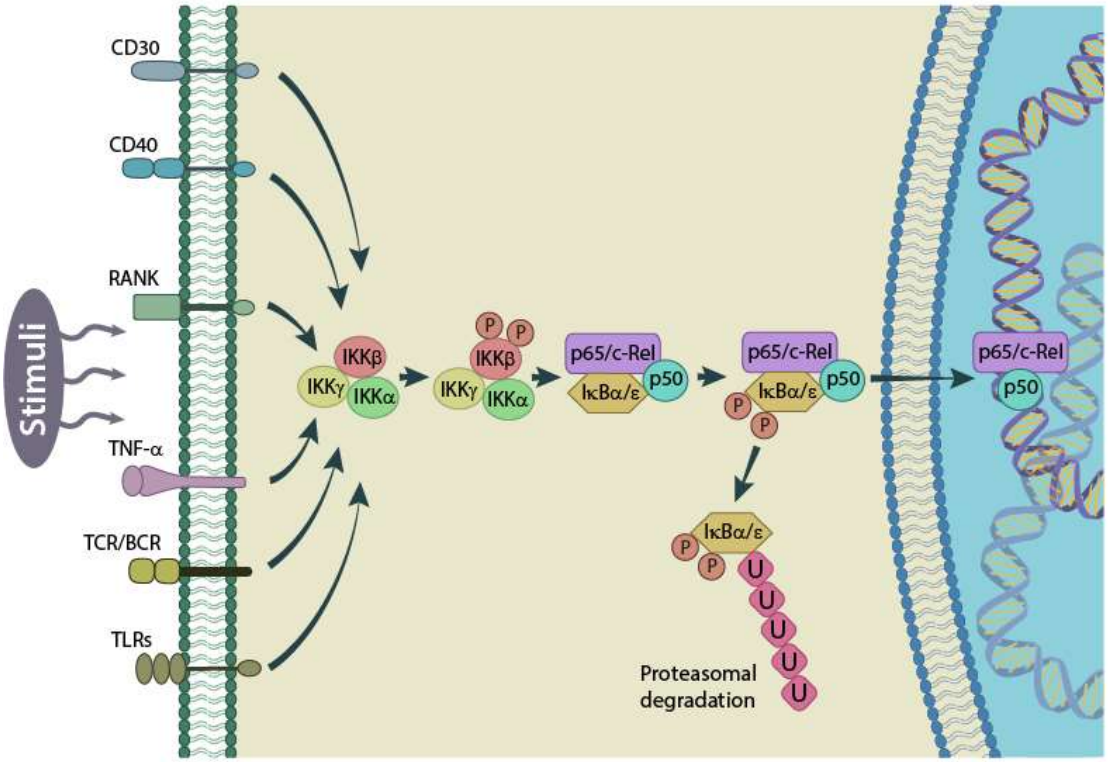

Fig. 3 Canonical NFκB pathway (https://www.mechanobio.info)

It was shown that, in a model of systemic inflammation, astrocytes from animals produce elevated levels of TNF-α, IL-1β, IL-6, IL-18, COX-2 as well as reduced levels of anti – inflammatory IL-10. This corresponded with upregulation of TLR2, NFκB and iNOS (Bellaver et al., 2017). Apart from the obvious stimulation of inflammatory response mediated by produced cytokines, inducible nitric oxide synthase (iNOS) - target of NFκB transcription, production of these molecules is highly associated with pathological conditions and is present in both astrocytes and microglia. Activity of iNOS is Ca^{2+} independent and subsequent generation of NO cause dysregulation of cellular redox state, increase in nitric reactive species and reduction of the ability to reuptake glutamate participating in glutamate excitotoxicity, which can lead to neuronal death (Yuste et al., 2015). Birck et al. performed microarrays experiments revealing changes in expression of 150 genes in astrocytic culture devoid of microglia after treatments with TNF-α. Genes that were upregulated included ones important for signaling via various cytokines (IL-10, 17, 15), Toll-like receptors (TLR5, 7,8,9), signaling via TNF-R2 or complement signaling. Both canonical and non-canonical pathways of NFκB signaling were upregulated as well as genes of proteins mediating posttranslational modifications of pathway members. Not surprisingly, pro-apoptotic and anti-apoptotic signaling was more active (Birck et al., 2016).

Communication between astrocytes and microglia

After injury to the CNS or in infection, not only astrocytes are activated. Cross-talk between astrocytes and microglia is expected and has been documented. In fact, reactive microglial stimulation is necessary for astrocytic activation (Liddelow et al., 2017). Both cell types react to antigens in their environment and respond by production of number of proteins that can have paracrine or autocrine effects. However, the assumption that microglial activation involving NFκB pathway and typical production of pro – inflammatory proteins IL-6, IL-1β, TNF-α, iNOS, COX-2 leads to astrogliosis in the form of increase of intermediate protein GFAP is not as straightforward. Röhl et al. confirmed that treatment of 9 days old astrocyte cultures with conditioned media from microglial cultures activated by LPS and producing above mentioned proteins reduces GFAP by 50%. They made the same observation in older and established 32 days old cultures with stable GFAP levels, indicating that microgliosis is not the only prerequisite to astrogliosis. However, this treatment did elicit a 10-fold increase in IL-6 production in astrocytes confirming the cross-talk and importance of microglial – astrocytic interactions in pathological states (Rohl et al., 2007). These interactions may also play a role in neurodegenerative diseases as both cell types have been implicated. In another study, manganese was used to activate microglial inflammatory phenotype verified by deramification, increase in mRNA of Tnf, Il-1β, Il-6, Ccl2, Ccl5, which translated into elevation in TNF and IL-6 cytokines and CCL-2, RANTES chemokines. Co-culture with astrocytes or their treatment with microglial conditioned media resulted in astrocyte activation and production of TNF, IL-6, CCL-2, RANTES. This group then blocked NFκB pathway in microglia with IKK complex blocker Bay 11-7082 and repeated experiments showing that blocking NFκB pathway prevents the production of mentioned cytokines and reduces inflammatory response in Mn-induced toxicity (Kirkley et al., 2017).

NFκB in neurons

The role of NFκB pathway in neurons is diverse as it is involved in processes of neuronal development (Zhang et al., 2012), survival and apoptosis but also is associated with information processing, learning and memory (Pizzi et al., 2009; Shih et al., 2015). The level of NFκB activity is higher in the brain than in peripheral tissue with constitutive activity in glutamatergic neurons. Those include cortical neurons in layers 2, 4 and 5, granule cells and pyramidal neurons in CA1 and CA3 regions of the hippocampus. Rodent brains exert NFκB activity also in the cortex and hippocampus as

well as amygdala, cerebellum, hypothalamus and olfactory lobes. The most common NFκB dimers in the developing rat brain are c-Rel/p65, p50/p65 and p50/p50. Evidently, p50 and p65 are abundantly present in neurons and the p50/p65 dimer is constitutively active in the adult brain (Kaltschmidt et al., 1994; Shih et al., 2015).

NFκB pathway in neurons is activated by a number of ligands, such as TNFα, glutamate, NGF, activity dependent neurotrophic factor ADNF, amyloid precursor proteins, adhesion molecules and more. This pathway is active in pathological states induced by ischemia, traumatic injury, oxidative stress, and its transcription profile in neurons enhances survival with production of proteins like Bcl-2, Bcl-$x_{L,}$ IAPs, Mn-SOD, but also can include production of pro – apoptotic proteins (FASL) and TNFα, enhancing NFκB response (Mattson and Meffert, 2006). TNFR2 activated NFκB pathway promoted survival of cortical neurons subjected to excitotoxicity (Marchetti et al., 2004; Dolga AM, 2008). Stem cell factor (SCF) was also shown to elicit neuroprotection via p50/p65 NFκB pathway and production of anti - apoptotic genes Bcl-2, Bcl-x_L in models of apoptosis and excitotoxicity (Dhandapani et al., 2005). Pigment epithelium- derived factor (PEDF) is neuroprotective in cerebellar granule neurons inducing the activity of p65/p50 NFκB dimer, which can be verified by measuring decreased levels of IκBα proteins, an increase in IκBα phosphorylation as well as p65 nuclear translocation. Neuroprotection by PEDF was demonstrated in models of glutamate excitotoxicity or induced apoptosis by low K^+ levels, which was blocked when NFκB inhibitor Bay 11-7082 was used. Moreover, upregulation of anti – apoptotic genes Bcl-2, Bcl-x, Mn-SOD in immature neurons and growth factors NGF, BDNF and GDNF in both immature and mature neurons (Yabe et al., 2001). Lang et al. demonstrated that NFκB deficiency (p50(-/-) mice) is associated with disruption of intracellular calcium buffering, accelerated hearing loss and increased vulnerability to noise induced injury in aging mice, suggesting its involvement in neuroprotection against age related neurodegeneration (Lang et al., 2006). Constitutive activity of p50/p65, virally delivered into retinal ganglion cells was shown to reduce tissue damage a neural loss after optic nerve crush (Dvoriantchikova et al., 2016). However, Ishige et al. demonstrated that roles of different NFκB proteins vary in model of glutamate excitotoxicity using antisense oligonucleotides to discriminate response by individual NFκB proteins. They found that p65 exhibited pro – apoptotic effects in HT22 cells while p50 was anti – apoptotic (Ishige et al., 2005). Pizzi et al. demonstrated that in excitotoxic conditions, IL-1β protects neurons against death and activates NFκB

proteins p50, p65 and c-Rel as opposed to p50 and p65 when only glutamate was present. They showed, that c-Rel dimers promote neural survival, while p50/p65 dimers promoted neural death (Pizzi et al., 2002). Evidently, more work is needed to fully discriminate effects of NFκB dimers in and proteins under specific conditions as results seem to vary. There are studies suggesting that NFκB is directly involved in neuronal death in ischemia (Stephenson et al., 2000) (Ridder and Schwaninger, 2009) and indirectly, through the actions of glial cells (Mattson and Meffert, 2006). However, growing evidence suggests that NFκB pathway is crucial for cell survival and when inhibited or aberrantly active, it stimulates neurodegenerative processes (Fridmacher et al., 2003). That has been confirmed in a study made by Baiguera et al. where c-Rel has been demonstrated to be crucial in prevention of degenerative changes in dopaminergic neurons in substantia nigra pars compacta. They showed, that knockout mice (c-Rel$^{-/-}$) develop Parkinsonism at 18 months of age and dimers containing c-Rel can induce expression of Mn-SOD and anti-apoptotic Bcl-x_L (Baiguera et al., 2012).

1.4. Regenerative potential of spinal cord

Unlike the peripheral nervous system, the central nervous system displays very limited regenerative potential following traumatic injuries. A series of intrinsic pathways and extracellular factors prevent axons from growing back and reestablishing lost connections. Instead, Wallerian degeneration distally from severed axon occurs as well as axonal dieback by up to 800 μm leaving dystrophic end bulbs that persist at the lesion border (Filous and Schwab, 2018) (Fig. 4).

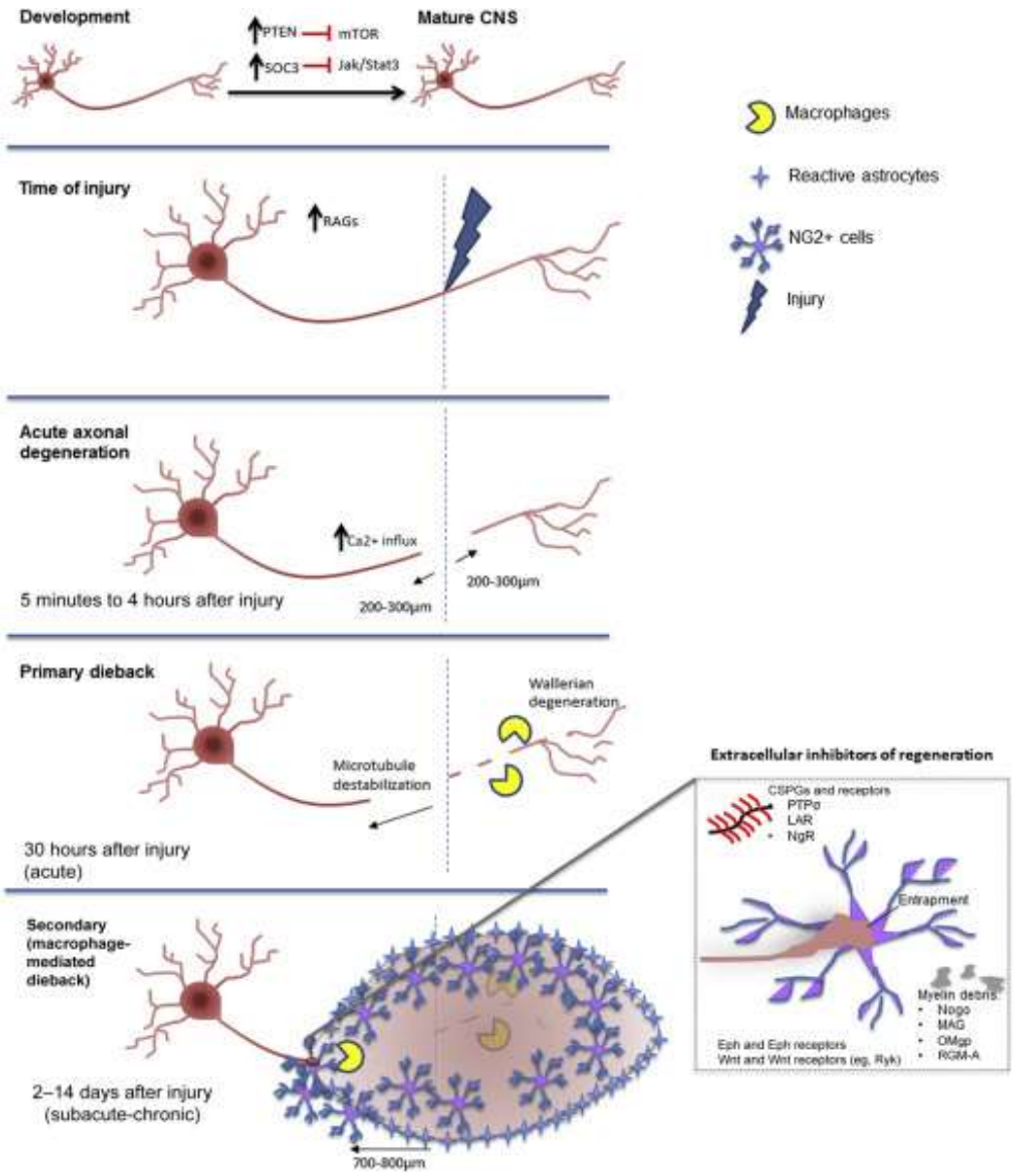

Fig. 4 Axonal degeneration after spinal cord injury (Filous and Schwab, 2018).

Infiltrating immune cells as well as resident microglia/macrophages are thought to contribute to axonal retraction and dieback (Busch et al., 2011). There is also evidence that macrophages can be beneficial in limiting of axonal dieback depending on receptor activation, i.e. TLR2 (Gensel et al., 2015). In healthy nervous tissue, axonal growth cones are active and can grow at a rate of 1 mm/day, however they stop growing and become dystrophic in the inhibitory environment of glial scar, which is made of chondroitin sulphate proteoglycans (CSPGs). Normal growth cones have been shown to contain organized microtubules, whereas dystrophic cones, if formed, are made of disorganized microtubules, with capability to form only short filopodia (Kerschensteiner et al., 2005; Erturk et al., 2007). Interestingly, transcription of

regeneration associated genes (RAGs) is induced after injury, via Ca^{2+} signaling. Several transcription factors, such as Kruppel-like factor 7 (KLF7), SRY-related HMG-box 11 (SOX11), Signal transducer and activator of transcription 3 (STAT3), are activated and initiate transcription of RAGs (Venkatesh and Blackmore, 2017). These genes include *Gap43,* brain abundant membrane attached signal protein 1 (*Basp1/Cap23), Arg1,* ciliary neurotrophic factor (*Cntf),* leukemia inhibitory factor *(Lif)* (Sun and He, 2010), but their expression is insufficient in enhancing axonal regeneration, which is largely inhibited by extracellular factors. After injury, $NG2^{+}$ glia, macrophages and pericytes surround the lesioned area and for the core of glial scar, while the penumbra of glial scar is mostly made of reactive $GFAP^{+}$ astrocytes (Rao and Pearse, 2016; Filous and Schwab, 2018). It has been shown that neurons form synapse-like connections with neuron-glial antigen 2^{+} ($NG2^{+}$) glia, which could prevent further axonal dieback, but also regeneration (Filous et al., 2014). Components of the glial scar are strongly inhibitory to axonal growth, but its absence has been shown to prevent regrowth as well. This has been suggested to be due to absence of growth stimulating molecules produced by activated astrocytes (Anderson et al., 2016). Several extracellular inhibitory factors have been identified that contribute to the non-permissive environment. Examples of such molecules are Nogo-A, Tenascine R, repulsive guidance molecule A, semaphorins and ephrins, acting as inhibitory and repulsive guidance molecules (Filous and Schwab, 2018) (Fig. 4).

1.5. Phytochemicals

A growing number of studies focus on neuroprotective properties of various phytochemicals. These compounds are typically polyphenols, containing aromatic ring with one or more hydroxyl groups naturally found in plants (EGCG, olive oil, luteolin) or their fruits (resveratrol, curcumin, Withaferin A) and have been found to be beneficial in treatment of secondary injuries after trauma to the CNS. These substances exert anti-oxidant properties protecting tissues from lipid peroxidation, anti-inflammatory properties taming inflammation after injury and promoting healing as well as promoting production of growth factors. They have also been found to have anti-apoptotic effects and can inhibit expression of Nogo-A (Khalatbary, 2014; Gundimeda et al., 2015; Yan et al., 2017).

1.5.1. EGCG

Epigallocatechin-3-gallate is a polyphenol found in green tea that has been shown to have anti-inflammatory, anti-apoptotic, anti-edema effects as well as properties stimulating regeneration or reducing the presence of inhibitory molecules in the environment after spinal cord injury (Tian et al., 2013; Khalatbary, 2014; Gundimeda et al., 2015) (Fig 5). EGCG does reach the brain and spinal cord at approximately 20x lower concentration than that in plasma, however low concentrations are potent enough to prevent or slow the accumulation of cognitive impairments (Pervin et al., 2017). EGCG is also a known anti-oxidant and its effects could partly be due to free radical scavenging properties (Biasibetti et al., 2013).

Fig. 5 Epigallocatechin-3-gallate (Furst and Zundorf, 2014).

Treatment with EGCG after SCI has been shown to inhibit the inflammatory response by reducing the numbers of infiltrating neutrophils and levels of TNF-α, IL-1β, COX-2, iNOS, which can all contribute to prolonged inflammation. Moreover, administration of EGCG led to upregulation of anti-inflammatory cytokine IL-10 and inhibition of TLR4 (Khalatbary and Ahmadvand, 2011; Kuang et al., 2012). It has also been shown that EGCG has anti-apoptotic as well as regenerative properties as it alters the ratio of pro-apoptotic and anti-apoptotic proteins Bax and Bcl-2 in favor of Bcl-2 and it also promotes expression of GAP-43, protein expressed in sprouting axons (Renno et al., 2015) or growth factors BDNF and GDNF (Tian et al., 2013).

1.5.2. Curcumin

Curcumin is a polyphenol compound found in common spice turmeric, which is produced from the rhizome of *Curcuma longa* plant (Fig. 6). A large number of studies have been produced documenting its beneficial properties in reducing inflammation, apoptosis, glutamate induced toxicity or its anti-oxidant and anti-cancer potential (Chang et al., 2014; Yao et al., 2015; Gokce et al., 2016; Sanivarapu et al., 2016; Lodi et al., 2017).

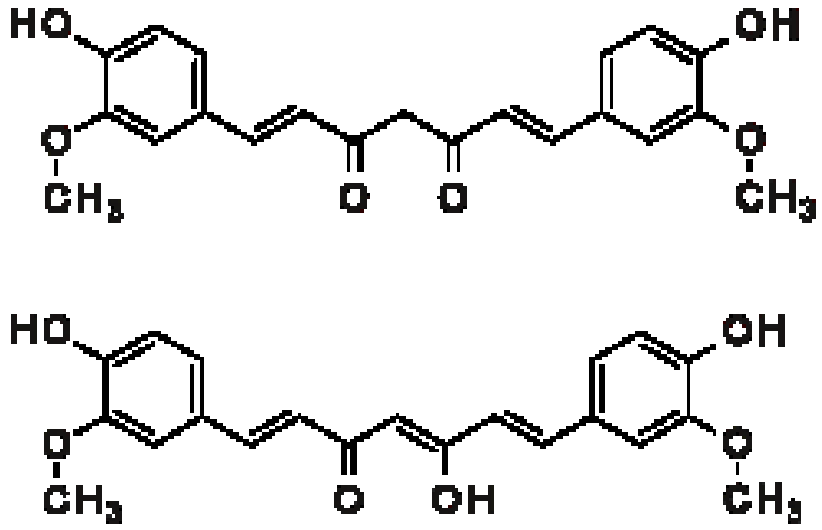

Fig. 6 Curcumin keto- and enol- form.

Curcumin is known to have anti-oxidant and anti-inflammatory effects in treatment of secondary injury after SCI. Curcumin reduces levels of oxidant malondialdehyde (MDA) and increases levels of anti-oxidant enzyme superoxide dismutase (SOD). Curcumin also reduces levels of the common cytokines and chemokines involved in the inflammatory response such as TNFα, IL-1β and reduces activities of transcription factors NFκB, SOX-9 resulting in decrease of glial scar formation (Yuan et al., 2017), or it alters the mammalian target of rapamycin (mTOR) signaling pathway, which is involved in cellular metabolism, autophagy or neural development (Lin et al., 2017).

1.6. Cell transplantation in treatment of spinal cord injury

A lot of research has focused on testing various types of stem or progenitor cells as treatment of spinal cord injury in efforts to enhance neuroprotection, axonal and myelin regeneration and immunomodulation. Stem cells are characterized by their capability to

differentiate into various cell types and to renew themselves. Embryonic stem cells (ESC) are pluripotent and differentiate into all three germ cell layers, while adult stem cells residing in many tissues are either multipotent or unipotent giving rise to particular lineages or cell types. These include mesenchymal stem cells, neural stem cells, liver stem cells, dermal stem cells and many more (Oh and Jeon, 2016; Singh et al., 2016; Assinck et al., 2017; Bouvier et al., 2017; Gonzalez et al., 2017).

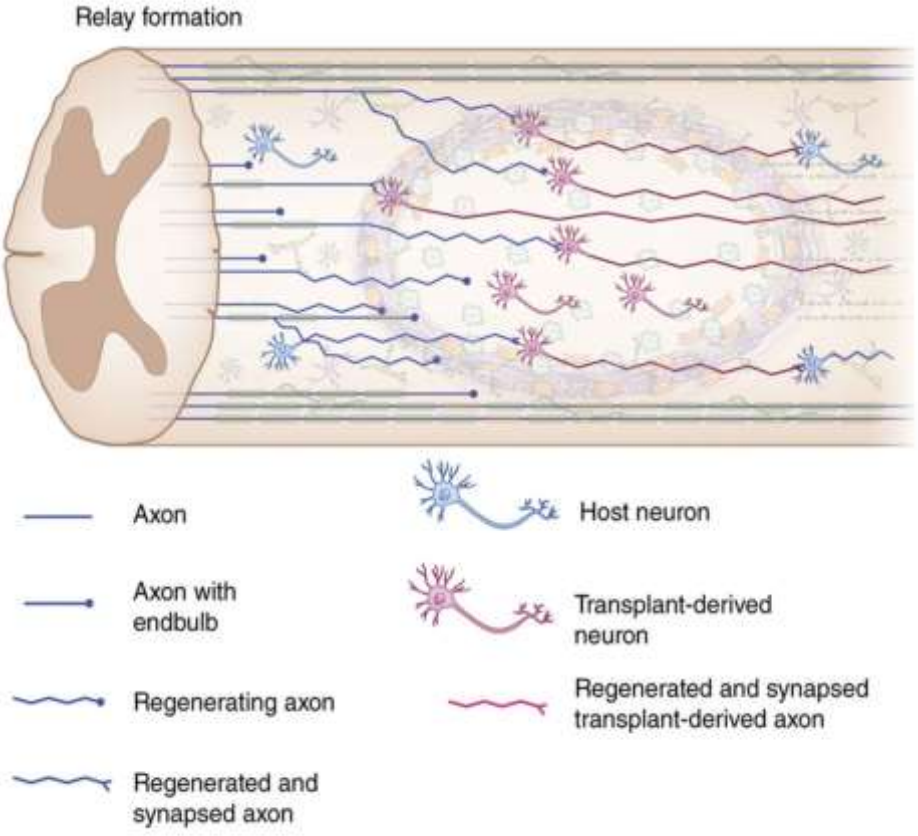

Fig. 7 Relay formation after cell therapy (Assinck et al., 2017).

It has been shown, that transplantation of cells can result in greater tissue sparing, mostly of white matter, which could in some cases, be due to production of myelin by grafted cells. Enhanced preservation of spinal cord tissue tends to correlate with improved functional outcome (Assinck et al., 2017). One of the mechanisms of how cell therapy contributes to motor improvement is partially bridging lesion cavity and providing physical support for host axons to grow on, which may further be enhanced by laminin produced by grafted cells. This effect of transplanted cells can be enhanced by combined therapy with biomaterials (Lai et al., 2016; Fan et al., 2017; Liu et al., 2017; Xu et al., 2017). Observations suggest that transplanted cells with similar origin

as target tissue provide better axonal growth support (Assinck et al., 2017). This approach appears to be more efficient when combined with a mix of growth factors enhancing the ability of transplanted cells to differentiate into cells capable of relaying transmission (Lu et al., 2012; Zhu et al., 2018) (Fig. 7). Transplanted cells can produce a number of cytokines and growth factors, as shown *in vitro*, which can have beneficial effects on spinal cord regeneration and host cell survival. Secreted factors may improve blood vessel regeneration as well as modulate immune reaction and glial scar formation (Assinck et al., 2017).

1.6.1. Bone marrow mesenchymal stem cells

Bone marrow mesenchymal stem cells (MSCs) (Fig. 8) are multipotent cells capable of differentiating into several different cell types such as adipocytes, chondroblasts, and osteoblasts (Fu et al., 2017; Gupta et al., 2017; Saler et al., 2017). It has been shown, that MSCs are able to produce a variety of growth factors like BDNF, GDNF, NGF, hepatocyte growth factor (HGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), which can promote neurite outgrowth *in vitro*, but also promote regeneration *in vivo* (Lin et al., 2014; Qu and Zhang, 2017). Moreover, transplantation of MSCs causes host transcriptional changes, which in turn modulates processes taking place after spinal cord or stimulates regeneration (Torres-Espin et al., 2013). Mesenchymal stem cells alter cytokines level, decreasing TNFα, IL-1β, associated with stimulation of inflammation and tissue loss (Han et al., 2015). They have also been shown to have anti-apoptotic effects via downregulation of caspase 3 and by changing of the Bax/Bcl-2 ratio (Yin et al., 2014; Gashmardi N1, 2017). Transplantation of MSCs alone improves functional recovery in rodent models of SCI (Pal et al., 2010; Wright et al., 2011) or dogs with naturally occurring SCI (Penha et al., 2014). A number of studies used MSCs in combination with biomaterial or with growth factors to further improve outcomes and survival of grafted cells. A study utilizing 3D gelatin sponge coated with poly-(lactide-co-glycolide) seeded with MSCs resulted in higher levels of angiogenesis, reduced cavity formation and reduced levels of TNFα, IL-1β (Zeng et al., 2011). The use of natural acellular scaffold in combination with MSCs promoted functional recovery and reduction in apoptosis (Chen et al., 2014). Several studies evaluated effects of genetically modified MSCs expressing growth factors neurotrophin-3 (NT-3) or basic fibroblast growth factor (bFGF) on spinal cord injury. Combination of MSCs with bFGF promoted axonal regrowth, which correlated with improved motor

function (Liu et al., 2011). Also in the case of NT-3 expressing MSCs, authors found that treatment with these cells improved motor function, reduced lesion cavity size, promoted axonal growth and reduced astroglial reaction (Wang et al., 2014). NT-3 transfected MSCs have also been shown to display their improved survival after transplantation as well as the survival of present motor neurons (Dong et al., 2014).

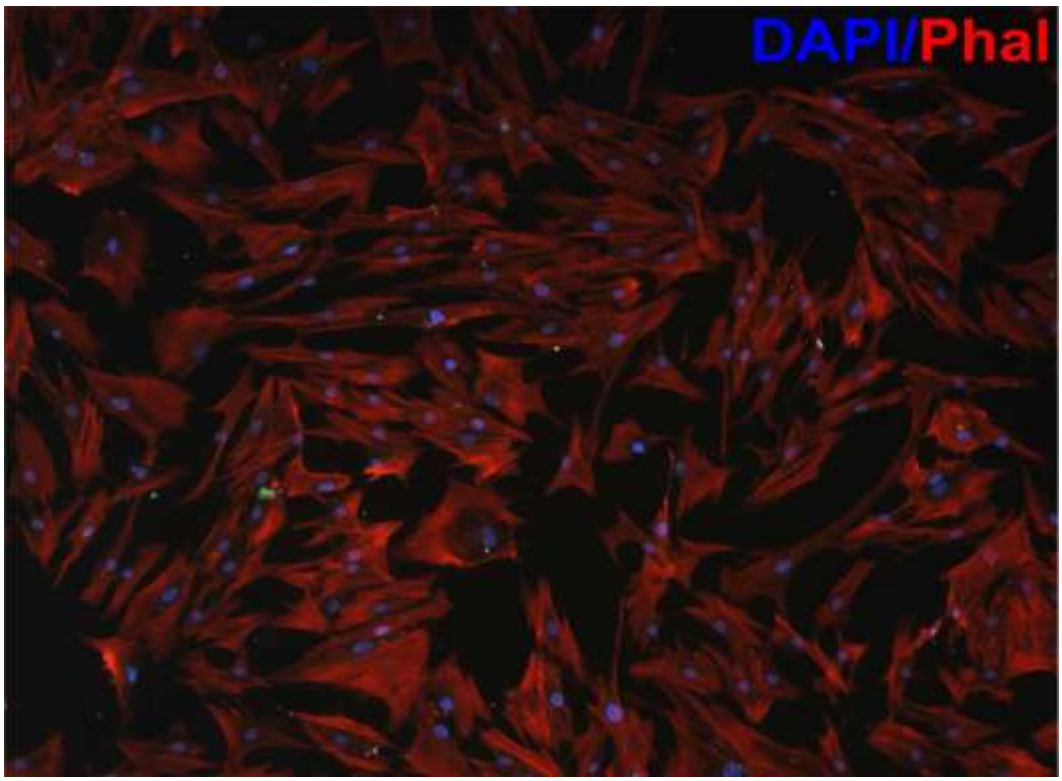

Fig. 8 Bone marrow mesenchymal stem cells. Courtesy of PharmDr. Šárka Kubinová, PhD.

To avoid cellular transplantation, studies using conditioned medium have emerged as safer alternative with promising results. Cantinieaux et al. showed anti-apoptotic and pro-angiogenic effects *in vitro* as well as *in vivo* with improved functional recovery supporting the notion that MSCs act predominantly via paracrine effects (Cantinieaux et al., 2013). Use of this type of cells has been of grave interest, confirmed by a number of clinical trials, because they are relatively easy to obtain and have been shown to be safe to use. They have also been proposed to elicit limited immune reaction of the host since they express low levels of major histocompatibility complex class I (MHCI) and do not express co-stimulatory molecules needed for lymphocyte activation (Torres-Espin et al., 2015). Unfortunately, a phase III clinical trial revealed only limited therapeutic effect of

MSCs injected intramedullary and subdurally, suggesting the need for trial with multiple MSCs injections (Oh et al., 2016; Fan et al., 2017).

1.6.2. Neural stem cells and neural precursors

Neural stem cells (NSCs) are multipotent and are known to be present throughout the adult mammalian central nervous system (Stenudd et al., 2015). However, NSCs that give rise to new neurons are generated in 2 regions of adult mammalian brain - in the subventricular zone (SVZ) and in the subgranular zone of hippocampal dentate gyrus (DG) (Eriksson et al., 1998; Eliwa et al., 2017). New NSCs are formed every day, they migrate and differentiate to form new connections replacing some of the neurons in olfactory bulb or hippocampus and integrating into existing circuitry. In humans, the number of newly formed neural stem cells is large, however less than 1% of mostly interneurons is replaced in olfactory bulb every 100 years implying limited functional significance (Klein and Fishell, 2004; Ihunwo et al., 2016). Interestingly, it was demonstrated, that NSCs generated in the SVZ migrate to striatum instead of olfactory bulb. The renewing fraction of interneurons amounted for 25% out of which 2.7% is turned over each year. Analysis of brains from patients with Huntington´s disease revealed strongly impaired postnatal generation of neurons as well as significantly reduced turnover of oligodendrocytes when compared with healthy control brains (Ernst et al., 2014). In hippocampi, about 700 new excitatory glutamatergic neurons (granule cells) are replaced every day. Not all populations in the hippocampus are replaced resulting in 1.75% annual replacement rate within the renewing fraction. Interestingly, neurons generated in adult neurogenesis are more likely to die off than neurons generated during development with 10x shorter half-life of 7.1 years (Spalding et al., 2013; Ihunwo et al., 2016). Neural stem cells can be cultured and expanded in vitro in the presence of growth factors, typically epidermal growth factor (EGF) and FGF (Fig.9). They can be passaged and form neurospheres, keeping their multipotency. NSCs start to differentiate into astrocytes, oligodendrocytes and neurons upon withdrawing growth factors from medium (Klein and Fishell, 2004). After expansion and transplantation of NSCs into neurogenic areas (SVZ, DG), they have been shown to integrate, differentiate and receive functional synaptic input. However, when transplanted into other parts of CNS, such as the spinal cord, they differentiate mainly into glial cells like astrocytes (Klein and Fishell, 2004; Amemori et al., 2013) .It has been shown, that adult neural stem cells isolated from the brain SVZ region survive well

in the lesion site in monkey spinal cords for at least 6 months, express neuronal marker Tuj1 and significantly improve locomotor function (Nemati et al., 2014). Hwang et al. discovered that physical training on treadmill improved the survival rate of grafted cells more than 3 fold in the first week and by 5 fold at 3 and 9 weeks after transplantation in rats. His group also identified that insulin-like growth factor-1 is behind this enhancement as its levels were significantly increased (and not of BDNF or NT-3) and when neutralized this effect on survival was lost. Additionally, they detected an increase in tissue sparing, myelination and level of serotonergic fiber innervation (Hwang et al., 2014). This indicates that more complex approach involving rigorous physical rehabilitation is required for best results of spinal cord injury therapy involving cell transplantation. Therapeutic effect of neural stem cells in injured nervous system is not only substitutional and direct contact of grafted cells and host tissue is not the sole mechanism by which improvements occur. It has been shown that continuous administration of neural stem cells conditioned medium resulted in the growth of corticospinal tract (CST) and tripling of synaptic formation between CST collaterals and propriospinal interneurons. Moreover, reduced levels of pro-apoptotic caspase 3 were detected as well as functional improvements (Liang et al., 2014). Adult progenitor cells exist in the spinal cord and when transplanted into spinal cord they also differentiate into glia. However, when transplanted into neurogenic areas of the hippocampus, they migrate and differentiate into neurons morphologically similar to granule cells, indicating that neural stem or adult progenitor cells are not lineage restricted and that local environmental cues are of high importance in cell differentiation (Shihabuddin et al., 2000).

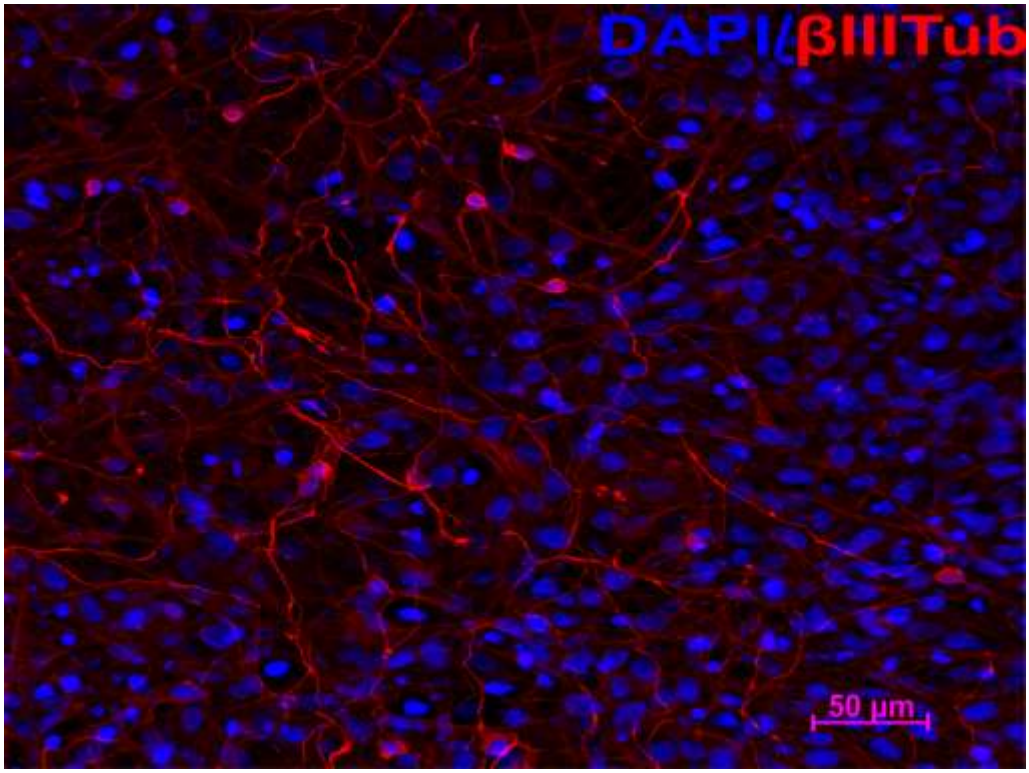

Fig. 9 A three week old culture of immortalized line of spinal neural precursors obtained from fetal spinal cord, SPC-01-GFP, stained for βIII-tubulin. Courtesy of Klára Jiráková, PhD.

The principal aims of therapies of spinal cord injury are to reduce inflammation and inhibitory effects of glial activation and to improve functional recovery by increased tissue preservation and neuroplasticity. Strikingly, only modest regeneration can result in major benefits as preserving of only about 10% of initial axon number can maintain function (Wilcox et al., 2014). Studies testing various neural stem or progenitor cells from different sources show mostly positive outcomes of varying magnitudes. Adult neural precursors isolated from the SVZ showed good survival after grafting and matured into oligodendrocytes, remyelinating axons and protecting white matter (Hawryluk et al., 2014; Wilcox et al., 2014), which was previously showed by Plemel et al. who used NPCs isolated from mouse fetal ventral forebrain. These cells were platelet-derived growth factor (PDGF) responsive and about 30% of surviving cells differentiated into oligodendrocytes. Detected oligodendrocytes associated with myelin basic protein (MBP) were located in the vicinity of nodes of Ranvier suggesting their role in remyelination of dysmyelinated Shiverer mice (Plemel et al., 2011). NPCs were also showed to reduce GFAP and CSPG positivity in the lesion site, which could have

led to the measured functional improvements (Hawryluk et al., 2014; Wilcox et al., 2014). Adult human spinal cord neural precursor cells have been shown to improve BBB score of transplanted rats exhibiting neuroprotective effect. These NPCs were transplanted as neurospheres and the majority of them differentiated into astrocytes and lower number into neurons (Emgard et al., 2014). Neural tube precursor were also found to improve functional outcome after SCI, they showed good level of integration, differentiated into cells of both glial and neural phenotype and contributed to decreasing the size of glial cyst (Boido et al., 2011). Bonner et al. demonstrated that transplanted mixed neural and glial restricted precursors are capable of forming neuronal relays. Combined with BDNF expressing lentivirus injected into dorsal column nuclei (DCN) as a cue to attract axons. This group detected robust axonal growth into DCN, presence of excitatory synapses between host regenerating axons and graft derived neurons as well as synapses between graft axons and DCN neurons. Presence of active synapses and functional transmission across relay connections was confirmed with electrophysiological recordings (Bonner et al., 2011). Neural precursor cells isolated from embryonic spinal cord and later transplanted into adult spinal cord white matter showed good survival and differentiated into astrocytes, oligodendrocytes and neurons similarly to the same cells transplanted into corpus callosum, however they migrated away from the injection site in spinal cord but did not move within or away from corpus callosum confirming crucial environmental cues depending on not only varying parts of the CNS but also varying locations of white matter (Jin et al., 2012).

1.6.3. iPS-derived neural precursors

In 2007, adult human fibroblast cells were reprogrammed into induced pluripotent stem cells (iPSCs) for the first time (Takahashi et al., 2007). Four transcription factors were previously identified by the same group to be sufficient to reprogram adult mouse fibroblasts (Takahashi and Yamanaka, 2006). Protocol for generation of human iPSCs differs from the one used to create mouse iPSCs, but the same transcription factors were introduced into human fibroblasts by retroviral transfection with plasmids encoding vesicular stromatitis virus glycoprotein (VSV-G) and the reprogramming octamer-binding transcription factor 3/4 (Oct3/4), (sex determining region Y)-box 2 (Sox2), Kruppel-like factor 4 (Klf4) and *c-myc*proto-oncogene (c-Myc) (Takahashi et al., 2007; Kim et al., 2017; Marote et al., 2018). iPSCs are similar to embryonic stem cells in morphology, surface antigens, gene expression and high telomerase activity. They

possess ESC-like pluripotency and proliferation capacity. Generated iPSCs expressed undifferentiated ESC marker genes, i.e. OCT3/4, SOX-2, growth differentiation factor 3 (GDF-3), NANOG, FGF-4, zinc finger protein 42 (REX-1/zfp42) and differentiated into all three germ layers *in vitro* and formed teratomas *in vivo* (Takahashi et al., 2007). Alternative methods have been described using a different set of reprogramming factors NANOG, LIN 28 or L-myc (Yu et al., 2007; Polentes et al., 2012; Capetian et al., 2016) (Fig. 9) or non-integrating episomal plasmids encoding OCT4, SOX2, KLF4, L-MYC, LIN28 and *p53* short hairpin RNA (shRNA) (Sareen et al., 2014). Several studies have been conducted to test effects of pre-differentiated neural stem cells, neural progenitor cells or neurospheres derived from iPSCs in the treatment of spinal cord injury. Kobayashi et al. implanted iPSC- derived neural stem cells 9 days after SCI in common marmosets. They observed significant improvements in motor function, which was measured with open field test, bar grip test and cage climbing test. Transplanted cells displayed good level of survival and differentiated into all three neural lineages – neurons, astrocytes and oligodendrocytes. Of all detected transplanted cells, nearly 99% expressed βIII-tubulin and 52% expressed a marker of mature neurons neuronal nuclear antigen (NeuN), 31% of transplanted cells were $GFAP^+$ and 26,5% expressed oligodendrocyte transcription factor 1 (Olig-1). About 24% expressed nestin, a marker of immature neural precursor cells. Additionally, enhanced axonal sparing/regrowth was observed as well as angiogenesis (increased numbers of platelet endothelial cell adhesion molecule 1 (PECAM-1) positive blood vessels) and prevention of demyelination (Kobayashi et al., 2012). Transplantation of iPSC-derived neurospheres 9 days after SCI results in good survival, integration within spinal cord Nori et al. (2011); (Sareen et al., 2014; Romanyuk et al., 2015) and differentiation into all three neural lineages (Nori et al., 2011; Tsuji et al., 2011; Romanyuk et al., 2015) with varying ratios neurons-astrocytes-oligodendrocytes. Okano´s group achieved lower survival rate of transplanted cells and 6 weeks after transplantation, they observed 30% of neurons, 50% of astrocytes and 14% of oligodendrocytes within the graft. In their study, they found enhanced level of serotonergic (5-hydroxytryptamine positive/$5\text{-}HT^+$) innervation of distal cord, reduced demyelination as well as significantly improved functional recovery (Tsuji et al., 2011). Nori et al. also transplanted iPSC-derived neurospheres 9 days after SCI and 8 weeks after they detected around 23% of mature neurons and 50% of $\beta\text{III-tubulin}^+$ neurons. This high portion of neurons was completed with 17% of astrocytes, 3% of oligodendrocytes and 10.7% of nestin^+ immature precursor cells. Most

of the mature neurons were GABAergic and only a small number of neurons were found to be serotonergic (5-HT$^+$) or cholinergic motor neurons (choline acetyltransferase positive/ChAT+). Moreover, newly formed synapses between graft and host neurons were detected as well as increased angiogenesis and myelination, which taken together resulted in improvement in motor function (Nori et al., 2011). Romanyuk et. al injected pre-differentiated iPS-derived neural precursor cells into spinal lesion 1 week after lesion induction and found 10% survival of grafted cells for up to 17 weeks. Strong endogenous neurofilament ingrowth was detected as well as positivity to NF200 and NF70 in grafted cells. Over time, large portion of the graft expressed GFAP. Despite GFAP prevalence, calbindin$^+$ interneurons, dopaminergic, serotonergic neuron and motor neurons were found along with significant functional improvements (Romanyuk et al., 2015). Several studies, however, demonstrated that transplantation of iPSC-derived neural progenitor cells did not result in functional recovery. Pomeshchik et al. observed no therapeutic effect of transplanted iPSC-derived neural progenitor cells with regard to cavity size and behavioral assessment. In this study, only 1 animal out of 6 showed any surviving grafted cells 6 weeks after transplantation indicating their loss over the course of experiment, hence no therapeutic effect. Authors suggested this was due to insufficient immunosuppression using only tacrolimus (Pomeshchik et al., 2015). However, other groups reported insignificant functional improvements while donor cell survival rates were good, as well as integration and maturation into all neural lineages (Nutt et al., 2013; Lu et al., 2014). Although in case of Nutt et al., low number of transplanted cells expressed NeuN and 50% expressed astroglial marker GFAP with the remaining neural cells expressing more immature phenotype, which was likely the reason for limited treatment effect (Nutt et al., 2013). Strikingly, group of Tuszynski reported extremely robust axonal growth originating from grafted cells, which exhibited excellent survival and most of them (71%) expressed NeuN and also MAP-2 and Tuj1 neural markers three months after transplantation out of which only 4.4% were ChAT$^+$ motoneurons. Relatively small portion of grafted cells differentiated into astrocytes (17.7%) and no oligodendrocytes were identified. Thousands of axons were detected extending across the entire neural axis to the brain, even the olfactory bulb and caudally from graft to distal lumbar spinal cord, which amounted to more than 9 cm in length. Despite numerous synaptic connections between graft and host, no functional improvement was observed. Several possible reasons were given to explain the lack of effect, i. e. low number or *fully*

mature neurons, insufficient myelination of new graft axons, formation of synapses that were not functionally beneficial or insufficient levels of neurotransmitters (Lu et al., 2014). Mixed results of therapies aimed at spinal cord injury using iPSC-derived cells underline the need for more studies to be conducted and refined to fully unlock their potential.

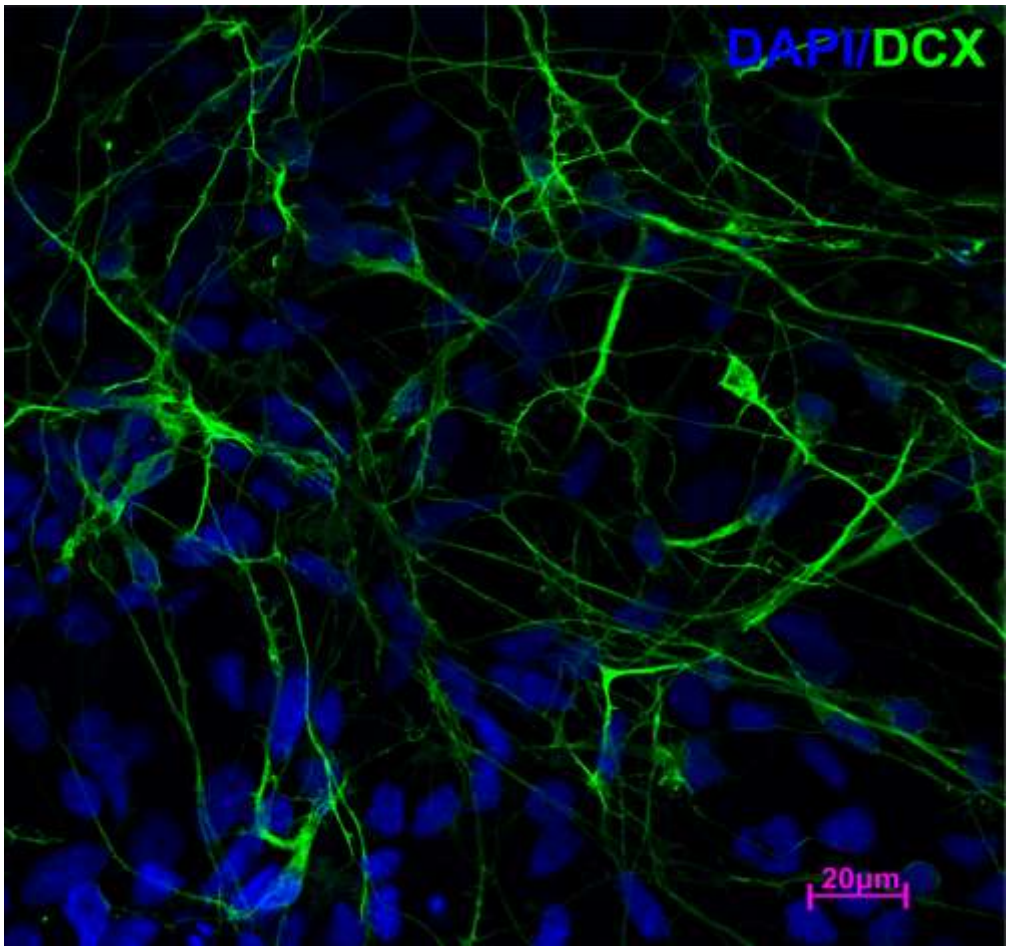

Fig. 10 Neural precursors derived from induced pluripotent stem cells (line iMR90) after 3 week differentiation expressing doublecortin (DCX). Courtesy of Klára Jiráková, PhD.

2. Hypothesis and aims

2.1. Hypotheses

1. We hypothesized, that NFκB may be activated after injury to the central nervous system. To investigate this, we measured p65 nuclear translocation at 6 different time points after inducing spinal cord injury.
2. We expected to see changes in NFκB activation after cell treatment of SCI as one of mechanisms of their effects on host tissue.
3. Curcumin and EGCG are phytochemicals found in the root of *curcuma longa* and in leaves of green tea, respectively. They are known to have both anti-inflammatory and anti-oxidant properties, which may influence the NFκB activity.
4. We hypothesized, that all three types of stem cells used in this study would have beneficial therapeutic effect, though their mechanism of action might be different. Mesenchymal stem cells have been thought to mostly act through production of various factors, which could lead to immunomodulation and enhanced regeneration. We expected that neural precursors obtained from fetal spinal cords or derived from iPS cells might interact with host tissue on a larger scale than MSCs leading to enhanced regeneration via direct contact, differentiation and thus replacement of lost cells.

2.2. Aims

1. To determine the pattern of activation of the NFκB (p65) canonical pathway after spinal cord injury in rats.
2. To investigate, how are the NFκB activity and inflammatory response modulated after SCI and subsequent transplantation with stem cells – mesenchymal stem cells, spinal neural precursors SPC-01, neural precursors derived from iPS cells.
3. To test effects of anti-inflammatory properties of curcumin and EGCG on NFκB activity and regeneration of spinal cords after injury.
4. To analyze regenerative potential of stem cells transplantation by determining their survival and differentiation potential in injured spinal cord and by measuring functional outcomes, axonal sprouting, effects of stem cells on the formation of the glial scar and changes in gene expression.

3. Methods

3.1. Animals

A total of 466 male Wistar rats weighing 270-300g were used in this study. Following spinal cord injury, they were randomly divided into groups surviving 1, 3, 7, 10, 14, 28 days or 9 weeks. Immediately after induction of SCI, rats were given anti-inflammatory compound curcumin (*n = 69*) or EGCG (*n = 53*), while control groups were given vehicle only (*n = 66, n= 53, respectively*). In experiments involving stem cells transplantation, rats were transplanted intrathecally (IT) with 5 x 10^5 MSC cells (*n = 47*) or saline (*n = 38*). Intraspinally (IS) transplanted were stem cells SPC-01-GFP (*n = 50*) or iPS-NPs (*n = 49*) with appropriate saline control (*n = 41*) on day 7.

All experiments were performed in accordance with the European Communities Council Directive of 22nd of September 2010 (2010/63/EU) regarding the use of animals in research, and were approved by the Ethics Committee of the Institute of Experimental Medicine ASCR, Prague, Czech Republic. The number of animals was statistically optimized for each particular experiment to achieve their reduction.

3.2. Spinal cord injury

The balloon-induced compression lesion model was developed to simulate the environmental situation and pathology of spinal cord tissue in the spinal canal, resembling the majority of spinal cord injury causes in humans (Vanicky et al., 2001).
To obtain reproducible and standard results, the weight and sex of the animals as well as the size of the balloon and the duration it is inflated must be kept constant among procedures. The ideal weight of male Wistar rats has been established as being in the range of 270 – 300 g (approx. 10 week old animals). Animals were anaesthetised with an inhalant mixture of isoflurane and air (3 v/v % isoflurane, AbbVie, Chicago, Illinois, USA), with an inflow of 300 ml/min, and kept under anaesthesia for the entire time of the procedure. After hair removal on the animal´s back, an incision was made at thoracic level. Carefully, vertabrae (T9, T10, T11) were exposed and laminectomy of the T10 vertebrae was performed. A 2 - French Fogarthy catheter was inserted into the epidural space through the created entry site. To produce a T8 lesion, the catheter was pushed in 1 cm and the balloon was then inflated with 15 µl of saline for 5 minutes.

Care was taken to ensure that no air bubbles were present in the balloon. After the lesion was complete, the catheter was deflated and removed and the wound was sutured in anatomical layers. Body temperature was continuously monitored with rectal thermometer and maintained at 37°C using a heating pad. Rats were given antibiotics (5 mg/kg, i.m., Gentamicin, Lek Pharmaceutical, Ljubljana, Slovenia) and analgesic (7.5 mg/kg, i.m., caprofen, Rimadyl, Phizer, New York City, NY, USA) for 10 days to prevent post-operative infection and to reduce pain. On day 6 (one day prior to transplantation), immunosuppression regime was started using Cyclosporine A (10 mg/kg i.p., Ciclosporin, Novartis, Basel, Switzerland) and azathioprine sodium (4 mg/kg, p.o. and 2 mg/kg p.o. after transplantation, Imuran, Aspen, KwaZulu-Natal, JAR) to prevent rejection. Animals were checked on daily and their urine was manually expressed twice daily until function was recovered, typically 5-8 days. Rats were housed in IVC cages with a 12 hour light/dark cycle and had unlimited access to water and food pellets.

3.3. Application of anti-inflammatory compounds

Animals selected for curcumin application were injected with 10 µl of curcumin (60 mg/kg, Sigma-Aldrich, St. Louis, MO, USA) diluted in olive oil in the vicinity of spinal cord injury through an opening created by laminectomy immediately after SCI. These animals were given curcumin daily for 1-28 days after injury (6 mg/kg i.p.) and again *in situ* once every 7 days, up to 4 times.

Animals used to study effects of EGCG were injected with 10 µl of EGCG in saline (50 mg/kg, Sigma-Aldrich, St. Louis, MO, USA) on the spinal cord surface immediately after induction of spinal cord injury and then every 7 days. Care was taken not to cause additional injury. Animals were also given 30µl of EGCG daily (50 mg/kg, Sigma-Aldrich, St. Louis, MO, USA).

3.4. Cell cultures

3.4.1. Mesenchymal stem cells

Human (h)MSCs were obtained based on the informed consent from patients enrolled in the clinical trial AMSC-DSD-001 and were provided by Bioinova Ltd. (Prague, Czech Republic). Cells were prepared under GMP conditions and supplied in 1.5 ml of cell suspension in Nunc (suspension of autologous MSC 3P). The mononuclear fraction containing hMSCs was separated from the bone marrow of healthy donors by gradient

separation using 25% Gelofusine (B. Braun, Melsungen, Germany). Cells were expanded in minimal essential medium (MEM) Eagle alpha media (Lonza, Basel, Switzerland), supplemented with 5% mixed allogeneic thrombocyte lysate (Bioinova Ltd.) and 10 µg/ml gentamicin (Lek Pharmaceuticals, Ljublanja, Slovenia). Cells (third passage) were analyzed using fluorescence-activated cell sorting (FACS) (LSR II; BD Biosciences, San Diego, CA, USA) and used for transplantation. Cells were positive for CD73, CD105, CD90, and major histocompatibility complex (MHC) class I, and negative for CD45, CD14, CD34, CD16, CD3, CD19, CD80, and MHC class II surface molecules. Control differentiation protocol (adipogenic, osteogenic, and chondrogenic) was performed to confirm the multipotent potential of the cells. Cell phenotyping was performed by Bioinova Ltd.

3.4.2. SPC-01

Human spinal cord cell line SPC-01 was produced from a 10-week old human fetal spinal cord. Obtained cells were conditionally immortalized with c-mycER^{TAM} construct and were also transduced with GPF using a lentivirus as vector. More detailed procedure description can be found here (Pollock et al., 2006). SPC-01 were grown in culture flasks coated with laminin (10 µg/ml, Sigma, Darmstadt, Germany) and media containing DMEM/F12 (Gibco, Life Technologies, Grand Island, NY), human apo-transferrin (100 µg/ml), human putrescine dihydrochloride (16.2 µg/ml), sodium selenite (40 ng/ml), human recombinant insulin (5 µg/ml), progesterone (60 ng/ml), L-glutamine (2mM), 4-hydroxy-tamoxifen (100 nM) all from Sigma, Darmstadt, Germany and human EGF and bFGF (20 ng/ml and 10 ng/ml, respectively) both from Peprotech EC, London, UK. Media was changed every 3rd day and cells were harvested at 60 – 70% confluence.

3.4.3. iPS-NPs

iPSC-NPs were prepared according to Polentes et al. (Polentes et al., 2012). In order to derive the human iPSC line, a lentivirus-mediated combination of OCT4, SOX2, NANOG, and LIN28 human cDNA (prepared plasmid from Addgene, Cambridge, MA, USA) was used for transduction of female (iMR90) human fetal lung fibroblasts (ATCC; Manassas, VA, USA). Clone selection, validation of the iPSC line, and derivation of neuronal progenitors are described in detail in the original article. Cells were routinely cultured in tissue culture flasks coated with laminin (10 µg/ml in

DMEM/F12) and poly-L-ornithine (0.002% in distilled water), both from Sigma-Aldrich (St. Louis, MO, USA). Growth medium comprising DMEM/F12 and Neurobasal medium (1:1), N2 supplement (1:100), B27 supplement (1:50; GIBCO, Life Technologies, Grand Island, NY, USA), L-glutamine (2 mM; Sigma-Aldrich, St. Louis, MO, USA), penicillin and streptomycin (50 U/ml; GIBCO), EGF (10 ng/ml), FGF (10 ng/ml), and brain-derived neurotrophic factor (BDNF; 20 ng/ml; PeproTech) was changed three times per week. A 7-day process of pre-differentiation in the same medium, except for the omission of FGF and EGF, was used prior to transplantation of iPSC-NPs.

3.5. Stem cells transplantation

Immortalized spinal neural precursors expressing green fluorescent protein (GFP) (SPC-01-GFP) or neural precursors derived from iPSC cells were injected in the lesion site 7 days after spinal cord injury. Animals were anaesthetised, fixed in a stereotactic frame (Stoelting CO, Wood Dale, IL, USA) and their spinal cords were carefully exposed at Th8. Using Nano-Injector (Stoelting CO, Wood Dale, IL, USA) and a glass pipette, 5 x 10^5 cells in 5 µl of saline were transplanted at a rate of 1 µl/minute. The glass pipette was kept in place for another 5 minutes to prevent backward leaking of cell suspension. Control animals received 5 µl of saline. In case after SCI, hMSCs (5×10^5/50 µl saline) were injected into the subdural space through the L5-L6 intervertebral space, while control animals received 50 µl of saline.

3.6. Immunohistochemistry

At days, 1, 3, 7, 10, 14, 28 days or 9 weeks after spinal cord injury (or 3, 7, 21 days or 8 weeks after transplantation), rats were transcardially perfused with 4% paraformaldehyde (PHA) for 15 minutes. Spinal columns were cut out and kept in 4 % PHA for 12 hours with subsequent dissection of spinal cords, which were kept in sterile phosphate buffered saline (PBS) until paraffin embedding. To monitor the survival, migration, and differentiation of transplanted stem cells, serial longitudinal sections of spinal cords (14 µm) were cut through the areas of interest, using a Leica CM1850 cryostat (Leica Microsystems GmbH, Vienna, Austria) 2 weeks (MSCs, $n = 4$; SPC-01, $n = 3$; iPSC-NPs, $n = 3$) and 2 months after transplantation. In case of MSCs, viable cells were visualized using long-term tracer carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) before implantation. SPC-01-GFP and iPS-NPs were visualized using

MTCO2 or Ku80 staining (both Abcam, Cambridge, UK). To determine survival as well as differentiation potential of transplanted stem cells and their interaction with the host tissue, histological and immunohistochemical (IHC) evaluation was performed either on longitudinal spinal sections [saline (IS + IT), $n = 7 + 6$; MSCs, $n = 11$; SPC-01, $n = 14$; iPSC-NPs, $n = 11$] or on cross-sections [saline (IS + IT), $n = 8 + 7$; MSCs, $n = 7$; SPC-01, $n = 8$; iPSC-NPs, $n = 10$] using antibodies against nestin (Millipore), glial fibrillary acidic protein (GFAP), βIII-tubulin (both Sigma-Aldrich, St. Louis, MO, USA), Nkx6.1 (DSHB, Iowa City, IA, USA), oligodendrocyte transcription factor 2 (Olig2), microtubule-associated protein 2 (MAP2; both Abcam, Cambridge, UK), and doublecortin (DCX; Santa Cruz Biotechnology, Heidelberg, Germany) were used. To visualize primary antibody reactivity, appropriate secondary antibodies were used: goat anti-mouse immunoglobulin G (IgG) conjugated with Alexa Fluor 488, as well as goat anti-rabbit IgG conjugated with Alexa Fluor 594 (Molecular Probes, Eugene, OR, USA).

Paraffin embedded spinal cords were cut into 5µm thick cross-sections. A total of 15 sections 1 mm apart were used from each animal amounting to 1,5 cm of analyzed tissue used for NFκB (p65) staining, followed by hematoxylin staining. Time points of 1-28 days ($n = 5$ for each group and time point) after SCI were used in this analysis. A total of 15 sections 1 mm apart were used from each animal amounting to 1.5 cm of analyzed tissue. To visualize the expression of p65, primary rabbit IgG antibody (Santa Cruz, Dallas, USA) and secondary goat IgG antibody conjugated with horseradish peroxidase H (Vector Laboratories, Burlingame, USA) were used. DAB (3′-diaminobenzidine) and H_2O_2 were added to produce brown precipitate where secondary antibody was present.

For morphometric and immunohistochemical analysis, spinal sections (15 cross sections, 5µm, 1 mm apart, total sample length was 1.5 cm) from animals surviving for 9 weeks ($n = 12$ for each group and time point) after SCI were used for Cresyl violet (0.25 g of cresyl violet dissolved in 100 mL of distilled water with 1 mL of 10% acetic acid) and Luxol-fast blue (1 g of Luxol-fast blue was dissolved in 100 mL of 96% ethanol with 5 mL of 10% acetic acid) staining to distinguish white and gray matter. To evaluate axonal sprouting and astrogliosis, primary antibodies against GAP43 (Millipore, Billerica, MA, USA) and GFAP (conjugated with cyanine dye (CY3), Sigma-Aldrich, St. Louis, MO, USA) were used. As a secondary antibody, goat anti-mouse IgG (conjugated with Alexa-Fluor 488, Abcam, Bristol, UK) was utilized.

3.7. Microscopy and image analysis

To estimate the number of implanted NPs (iPSC-NPs and SPC-01) 2 months after implantation, every sixth longitudinal spinal cord section (14-μm thickness) was chosen. Imaging of the whole graft area per section was performed (Observer D1 microscope; Carl Zeiss, Weimar, Germany). The surviving human cells were recognized using MTCO2 staining, and the analysis was performed using ImageJ software [National Institutes of Health (NIH), Bethesda, MA, USA]. The total number of human cells that survived was estimated according to the spinal cord volume in which the cells were found (number of MTCO2 sections multiplied by six). The percentage of surviving transplanted cells was calculated by dividing the estimated total number of surviving cells by the total number of transplanted cells (5×10^5). Data are presented as an average per transplanted group.

For NFκB (p65) nuclear translocation, microscope LEICA CTR 6500 with software FAXS 4.2.6245.1020 (TissueGnostics, Vienna, Austria) were used to take images of spinal cross sections. Analysis software HistoQuest 4.0.4.0154. (TissueGnostics, Vienna, Austria) was used for evaluation of the density of translocated p65 into cell nuclei per mm^2. Only DAB positive nuclei were included in the analysis. For each sample, 15 sections 1 mm apart were selected. Lesion centers were located on slide 8. For analysis of NFκB nuclear translocation, 3 regions of spinal cord injury were established – cranial, lesion center and caudal. There were 5 sections included in each region.

To measure the area of spared white and gray matter, Axioskop 2 plus microscope (Zeiss, Oberkochen, Germany) and ImageJ software (Wayne Rasband, NIH, Bethesda, MD, USA) were used. Volume of lesion cavities that developed in the area of spinal cord lesion 9 weeks after SCI was also assessed. Area of lesion cavities in serially sectioned spinal cords was measured from which the volume of cavity of individual spinal cords was calculated.

For immunohistochemical analysis of axonal sprouting (GAP43) and astrocytic reaction (GFAP), ZEISS AXIO Observer D1 microscope (Carl Zeiss, Weimar, Germany) was used. Either Wizzard (Carl Zeiss), or ImageJ programs were used for image analysis. Images were further processed with Excel (Office 2010, Microsoft) and CorelX6 (Corel Corporation) software. The number of $GAP43^+$ fibers per slide was manually counted. The graphical presentation of these results shows the average number of $GAP43^+$ fibers

per slide in percentage, when compared with the saline group, which was set to 100%. In analysis of $GFAP^+$ cells, the number of protoplasmic astrocytes per slice and the area of GFAP positivity surrounding the main lesion cavity were analyzed using ImageJ.

3.8. qRT-PCR

Expression of rat target genes *Sort1 (Nt-3), Fgf2, Olig2, Gap43, Gfap, Vegfa, Nfkb1, Cntf, Mip1α, Rantes, Cd86, Irf5* (M1 macrophage phenotype), and *Cd163* and *Mrc1* (M2 macrophage phenotype), 10 and 28 days after SCI with $n = 5$ per group per time point, was measured using a quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). RNA was isolated from paraformaldehyde-fixed spinal cord tissue sections, using the High Pure RNA Paraffin Kit (Roche, Penzberg, Germany). RNA was quantified with spectrophotometer (NanoPhotometerTM P-Class, Munchen, Germany), and isolated RNA was reverse transcribed into cDNA using Transcriptor Universal cDNA Master (Roche, Penzberg, Germany) and a thermal cycler (T100™ Thermal Cycler, Bio-Rad, Hercules, CA, USA). Reactions were performed using cDNA solution, FastStart Universal Probe Master (Roche, Penzberg, Germany) and TaqMan® Gene Expression Assays (Life Technologies, Carlsbad, CA, USA): glyceraldehyde 3-phosphate dehydrogenase/Gapdh/Rn01775763_g1, neurotrophin 3/Sort1/Rn01521847_m1, basic fibroblast growth factor/Fgf2/Rn00570809_m1, oligodendrocyte lineage transcription factor 2/Olig2/Rn01767116_m1, growth associated protein 43/Gap43/Rn01474579_m1, glial fibrillary acidic protein/Gfap/Rn00566603_m1, vascular endothelial growth factor A/Vegfa/Rn01511601_m1, nuclear factor of kappa B1/Nfkb1/ Rn01399572_m1, ciliary neurotrophic factor/cntf/ Rn00755092_m1, macrophage inflammatory protein 1α/Ccl3/Rn01464736_g1, chemokine (C-C motif) ligand 5/RANTES/Ccl5/Rn00579590_m1, CD86/Rn00571654_m1, CD163/Rn01492519_m1, interferon regulatory factor 5/Irf5/Rn01500522_m1, macrophage mannose receptor 1/Mrc1/Rn01487342_m1. Final reaction volume was 10 µL containing 25 ng of extracted RNA. Real-time PCR cycler (StepOnePlus™, Life Technologies, Carlsbad, CA, USA) was used for amplification. Following cycling conditions were used: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Relative quantification of gene expression was determined using the ΔΔCt method. Data was analyzed with StepOnePlus® software (Life Technologies, Carlsbad, CA, USA). For normalization of gene expression levels we used the *Gapdh* gene as a

reference. A log2 scale was used to display the symmetric magnitude for up- and down-regulated genes. From the obtained values of the control animals (SCI treated with vehicle), arithmetical mean was calculated and this value was set as zero. The statistical analysis was performed from ΔΔCt values of controls as well as treated animals.

3.9. Luminex

Levels of inflammatory cytokines in the spinal cord were determined 1, 3, 7, 10 and 14 days after SCI and application of anti-inflammatory compounds, stem cells or vehicle (*n = 5* per group, per time point). Fresh spinal cords were removed and kept on ice, then a 2 mm long segment from the center of the lesion was dissected and incubated in cell culture media (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) and 0.2% primocin for 24 h. Secretory cytokine levels were measured in the collected media. A customized Milliplex inflammatory cytokine kit (Millipore, Billerica, MA, USA) and Luminex instrumentation software were used to determine the levels of inflammatory cytokines. Rat cytokine Luminex custom eight-plex kits (for IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IFNγ, TNFα, MIP-1α) were used for customized bead assays. Assays were performed in 96-well filter bottom plates, according to the manufacturer's protocol. Antibody conjugated beads were used at a concentration of 5000 beads per marker and were incubated overnight with samples. Biotinylated detection antibody with streptavidin-RPE (streptavidin-R-Phycoerythrin) (Life Technologies, Carlsbad, CA, USA) was used to measure the levels of cytokines using Luminex xMAP 200 system (Luminex, Madison, WI, USA). Data was processed using Magpix instrumentation software. Mean fluorescence intensity (MFI) was used to calculate concentrations of individual cytokines; a four- or five-parameter logistic fit curve was generated for each cytokine from seven standards. The lowest standard, at least three-times above the background, was used to determine the lower limit of quantification (LLOQ). The LLOQ was calculated by subtracting the background (diluent) from the MFI of the lowest standard concentration and back-calculating the concentration from the standard curve.

3.10. BBB

The Basso Beattie and Bresnahan (BBB) test was used to evaluate hind limb locomotor performance (Basso et al., 1995). Treated (*n = 12*) or control (*n = 12*) rats were placed into circular arena and scored using 0-21 point scale. The BBB score reflects quality of

joint movement, paw placement, weight support, forelimb-hind limb coordination and trunk stability, 0 is assigned to no movement and 21 to normal performance (Tab. 2).

Score	Description
0	No visible movements of hind limbs.
1	Slight movement of 1 or 2 joints, typically of the hip and/or the knee.
2	Significant movement of 1 joint. Significant movement of 1 joint + slight movement of additional joint.
3	Significant movement of 2 joints.
4	Slight movement of all 3 joints.
5	Slight movement of 2 joints + significant movement of 3rd joint.
6	Significant movement of 2 joints + slight movement of 3rd joint.
7	Significant movement of 3 joints.
8	Sweeping motion without weight support. Plantar paw placement without weight support.
9	Plantar paw placement with weight support only when standing. Irregular, frequent or consistent gait on *dorsum pedis* with weight support and absence of plantar paw placement.
10	Irregular plantar paw placement with weight support but with no forward-backward coordination.
11	Frequent to consistent plantar paw placement with weight support but with no forward-backward coordination.
12	Frequent to consistent plantar paw placement with weight support with irregular forward-backward coordination.
13	Frequent to consistent plantar paw placement with weight support with frequent forward-backward coordination.
14	Consistent plantar paw placement with weight support and consistent forward-backward coordination. Paw rotates
15	Consistent plantar paw placement with weight support and consistent forward-backward coordination. No toe movements, paw is parallel to the body upon first contact and rotates upon lifting.

16	Consistent plantar paw placement with weight support and consistent forward-backward coordination. Frequent toe movements, paw is parallel to the body upon first contact and rotates upon lifting.
17	Consistent plantar paw placement with weight support and consistent forward-backward coordination. Frequent toe movements, paw is parallel to the body upon first contact and upon lifting.
18	Consistent plantar paw placement with weight support and consistent forward-backward coordination. Consistent toe movements, paw is parallel to the body upon first contact and rotates upon lifting.
19	Consistent plantar paw placement with weight support and consistent forward-backward coordination. Consistent toe movements, paw is parallel to the body upon first contact and upon lifting. Tail is on the ground part of the time/all the time.
20	Consistent plantar paw placement with weight support and consistent forward-backward coordination. Consistent toe movements, paw is parallel to the body upon first contact and upon lifting. Instability of the trunk. Tail is lifted consistently.
21	Consistent plantar paw placement with weight support and consistent forward-backward coordination. Consistent lifting of toes and tail, trunk is stable.

Tab. 2 Scoring of the open field BBB test (Basso et al., 1995).

3.11. Flat beam

Flat beam test was used to analyze complex motor function of rats after spinal cord injury. The apparatus consists of a 3.4-cm-wide and 140-cm-long wooden rectangular beam with a goal box placed at the end. Only the central 1 meter long part of the beam was selected for the motor performance evaluation. A video-tracking system (TSE-Systems Inc., Bad Homburg, Germany) recorded and measured the latency and trajectory of the rat to traverse the beam for maximum of 60 seconds. Animals were pre-trained before surgery inducing spinal cord injury, and then again before each testing, which started on the 3rd week after SCI. Rats were scored using the modified Goldstein

score (Goldstein et al., 1997) on a scale from 0-7, starting with no ability to balance and progressing to crossing the whole length of the beam properly using both hind limbs.

3.12. Plantar test

Hind paw withdrawal latency due to thermal stimulus was evaluated with specialized Plantar test device (Ugo Basile, Comerio, Italy). Rats were placed into a chamber and received thermal stimulus to the hind paws through a glass plate. Their withdrawal latencies were measured 5 times for each hind paw. The average of five values was used for statistical evaluation. Hyperalgesia was defined as a significant decrease in withdrawal latency.

BBB and plantar tests were performed prior to spinal cord surgery and then weekly after SCI.

3.13. Rotarod

Rotarod unit (Ugo Basile, Comerio, Italy) was used to test the motor function and forelimb-hind limb coordination of the rats. Rats were pre-trained before SCI. Speed of 5 rpm was used, and the latency to fall from the rotating rod was recorded.

Rotarod test and flat beam tests require more complex motor abilities of the rats, hence testing procedures started on the 3rd week after SCI and then on the 5th, 7th and 9th weeks after surgery.

3.14. Statistical analysis

Several statistical tests were used in our studies. Two-way repeated measurement (RM) ANOVA was always used to evaluate behavioral results. Two-way RM ANOVA was also used to test differences in spared gray/white matter and GFAP spatial distribution in addition to treatment effect. Differences in NFκB nuclear translocation between groups or location within injured spinal cords were tested using three-way ANOVA and two-way ANOVA with *post hoc* test Student, Neumann, Keuls (SNK) or with Bonferroni posttests. For immunohistochemical analysis of GAP43 and cavity size, one-way ANOVA or T-tests were used. Relative gene expression of genes related to regeneration and macrophage phenotypes and analysis of cytokine production were tested using two-way ANOVA. Statistical analyses were performed using either GraphPad Prism 5 (La Jolla, CA, USA) or Sigmastat 3.1 (Sistat Software Inc., San Jose,

CA, USA). Differences were considered significant * $p<0.05$, ** $p<0.01$, *** $p<0.001$. All data is shown as means ± standard error of the mean.

4. Results

The first and second section of results are based on data from listed articles:

Machova Urdzikova L, **Ruzicka J**, **Karova K**, **Kloudova A**, **Svobodova B**, **Amin A**, **Dubisova J**, **Schmidt M**, **Kubinova S**, **Jhanwar-Uniyal M**, **Jendelova P**. (2017) A green tea polyphenol epigallocatechin-3-gallate enhances neuroregeneration after spinal cord injury by altering levels of inflammatory cytokines. Neuropharmacology. Nov;126:213-223.

Machova Urdzikova L, **Karova K**, **Ruzicka J**, **Kloudova A**, **Shannon C**, **Dubisova J**, **Murali R**, **Kubinova S**, **Sykova E**, **Jhanwar-Uniyal M**, **Jendelova P**. (2015) The Anti-Inflammatory Compound Curcumin Enhances Locomotor and Sensory Recovery after Spinal Cord Injury in Rats by Immunomodulation. Int J Mol Sci. Dec 31;17(1).

Karova K, Machova Urdzikova L, Ruzicka J, Jhanwar-Uniyal M, Jendelova P. Transplantation of neural precursors generated from spinal progenitor cells reduce inflammation in spinal cord injury via NFκB pathway modulation. – Manuscript in preparation

4.1. NFκB pattern of activation in injured spinal cord

Our results of immunohistochemical analysis of spinal cords at 1, 3, 7, 10, 14 and 28 days after spinal cord injury indicate, that NFκB (p65) nuclear translocation is most pronounced in central areas of spinal cord lesions with mild increase cranially and caudally. NFκB (p65) nuclear translocation is elevated in the cranial and caudal parts of the injury and remains at similar levels in all time points (Fig. 11A). However, the course of p65 nuclear translocation in lesion center shows 2 peaks, first at 3 days after SCI and second at 28 days after injury reaching the highest value. We found strong NFκB activity at the last time point, which was significantly higher than all other time points with the exception of NFκB activity analyzed 3 days after induction of SCI. Moreover, NFκB translocation was found to be significantly higher at 3 days after SCI compared to 1 day interval (Fig. 11B).

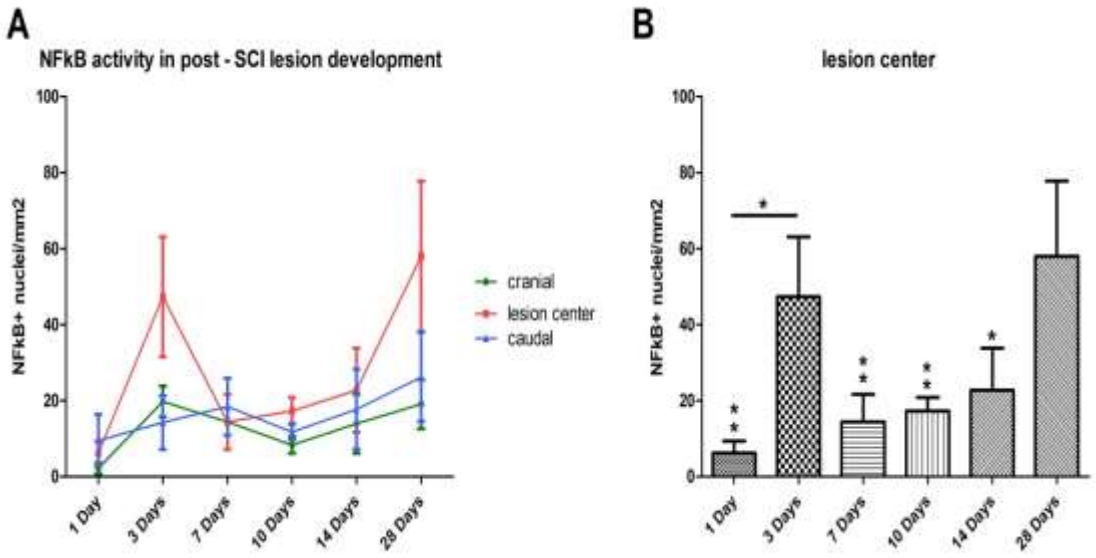

Fig. 11 Pattern of NFκB activity in lesion center and areas cranially and caudally from lesion center 1, 3, 7, 10, 14 and 28 days after injury, represented as number of $p65^+$ nuclei per mm^2. **(A)**. In the lesion center, significantly different to all other time points with the exception of 3 days after injury is data from group 28 days after SCI. NFκB related transcription is also significantly higher 3 days after injury when compared with 1 day interval **(B)**. Data is expressed as mean and the standard error of the mean (SEM) with $*p < 0.05$, $**p < 0.01$ indicating statistical significance.

4.2. Effects of EGCG or curcumin treatments on spinal cord injury

4.2.1. Modulation of NFκB activity

Density of NFκB $p65^+$ nuclei per 1 mm^2 was evaluated on transversal spinal cord sections in lesion centers and areas cranially and caudally at days 3, 7 and 28 after inducing spinal cord injury in animals treated with EGCG or saline and 1, 3, 7, 10, and 28 days after SCI in curcumin or vehicle treated animals.

We discovered that EGCG is strongly effective in decreasing NFκB activity in all tested time points when compared with controls. Translocation of $p65^+$ nuclei 3 days after injury was significantly reduced in the lesion center (Fig. 12A), whereas 7 days after injury such reduction was observed cranially to the lesion center (Fig. 12B). Moreover, activity of p65 associated canonical NFκB pathway was markedly reduced in all evaluated areas of spinal cord injury, cranial, central and caudal 28 days after SCI (Fig. 12C, Fig. 13A, A1).

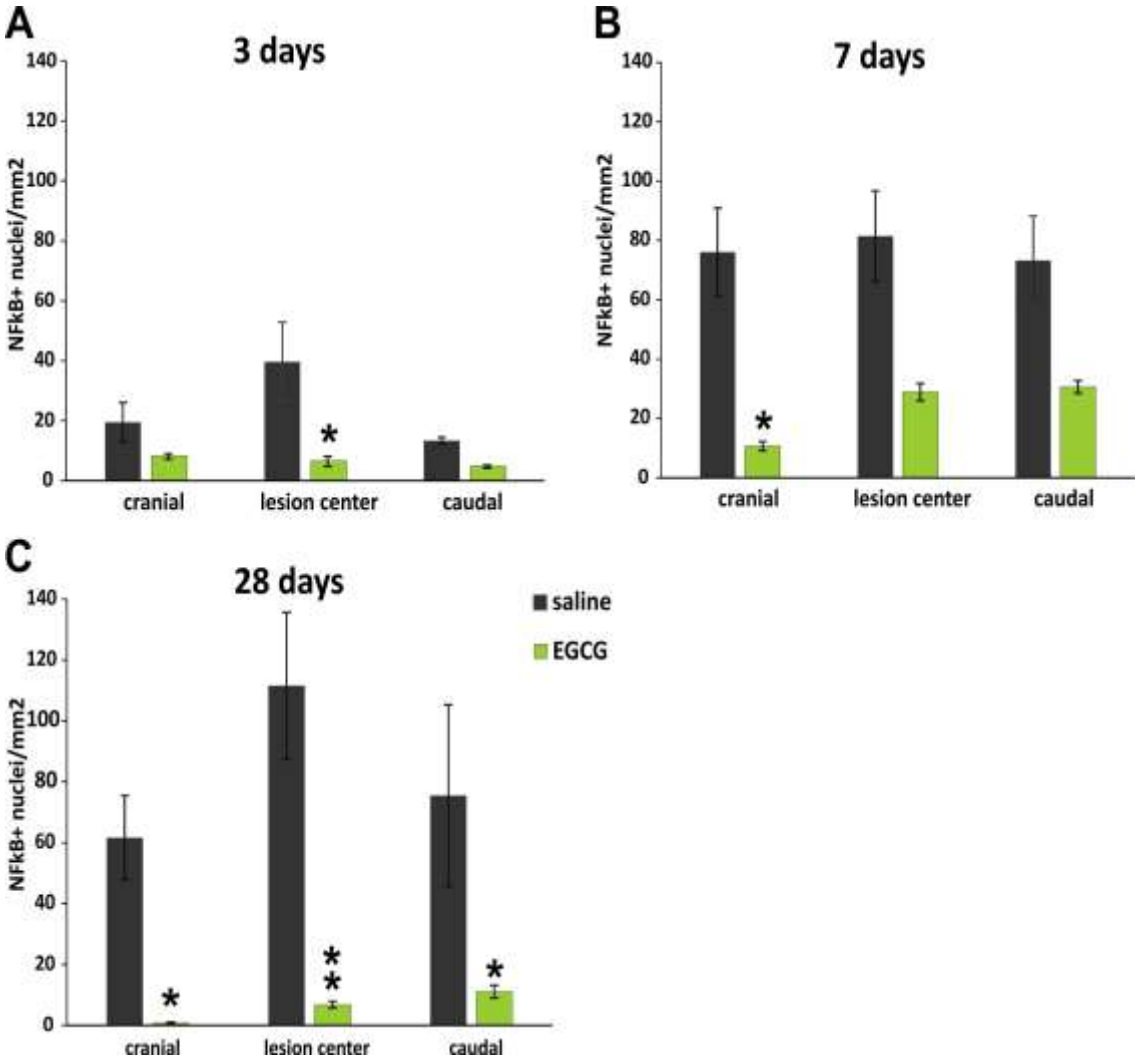

Fig. 12 Treatment with EGCG significantly reduced p65 associated NFκB pathway activation 3 days after SCI in the lesion center. Overall, the effect of EGCG was significant when compared to saline controls **(A)**. Significant reduction in p65 + nuclei density was observed 7 days after SCI, with a local significant difference in the cranial part of the spinal cord injury **(B)**. At 28 days after SCI, density of p65 + nuclei was further decreased and EGCG treatment proved to be highly significant **(C)**. Additionally, three-way ANOVA revealed an overall significance of EGCG in decreasing p65 + nuclei density. Data is expressed as mean and the standard error of the mean (SEM) with $*p < 0.05$, $**p < 0.01$ indicating statistical significance.

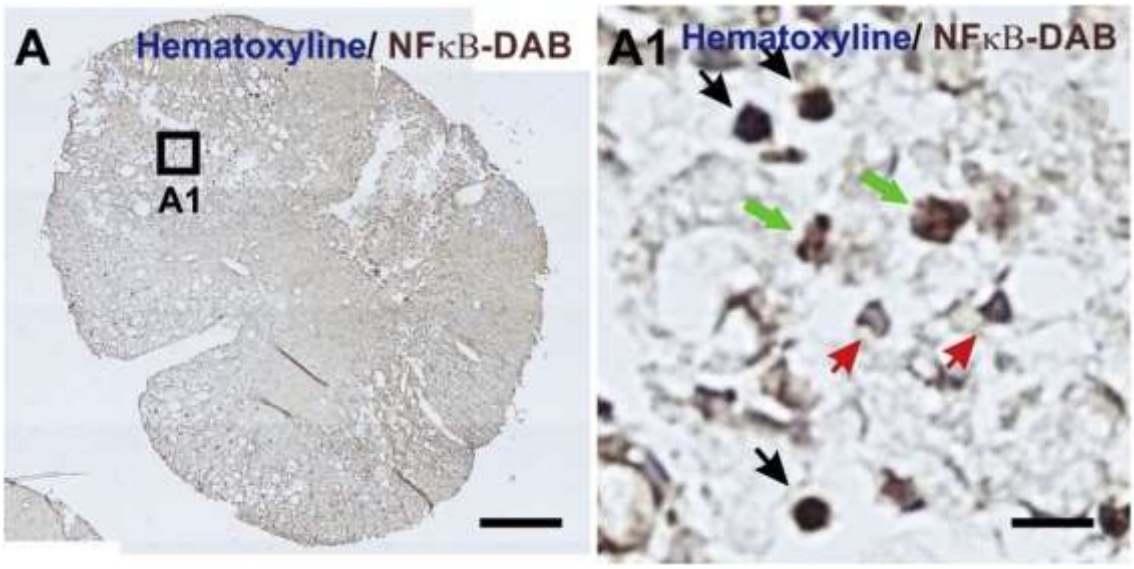

Fig. 13 Representative images show a section of the spinal cord lesion stained with hematoxyline and NFκB-DAB after EGCG treatment 28 days after SCI **(A)**. Black arrows indicate nuclei containing p65 (RelA) proteins of the NFκB dimer. In contrast, red arrows indicate $NF\kappa B^-$ nuclei stained with hematoxyline and cells with p65 in their cytoplasm highlighted with green arrows **(A1)**. Bar A = 500 μm, Bar A1 = 20 μm.

Similar results were obtained when analyzing spinal cords from animals treated with curcumin. In control animals, the highest number of NFκB-positive nuclei was observed at 10 days after SCI, followed by a slight decrease (Fig. 14d, E). Treatment with curcumin resulted in a significant reduction of NFκB activity at all studied time points when compared to control rats with the exception of 1 day after SCI (Fig. 14A), when only a local reduction was seen caudally from the lesion center. At the other time points, curcumin treated rats displayed a marked reduction in NFκB $p65^+$ nuclei, especially in the central parts of the injury (Fig 14B, C, D, E). At later time points (10 and 28 days after SCI), translocation of p65 into cell nuclei was essentially prevented by curcumin treatment in all evaluated areas of the spinal cord tissue (Fig. 14 D, E).

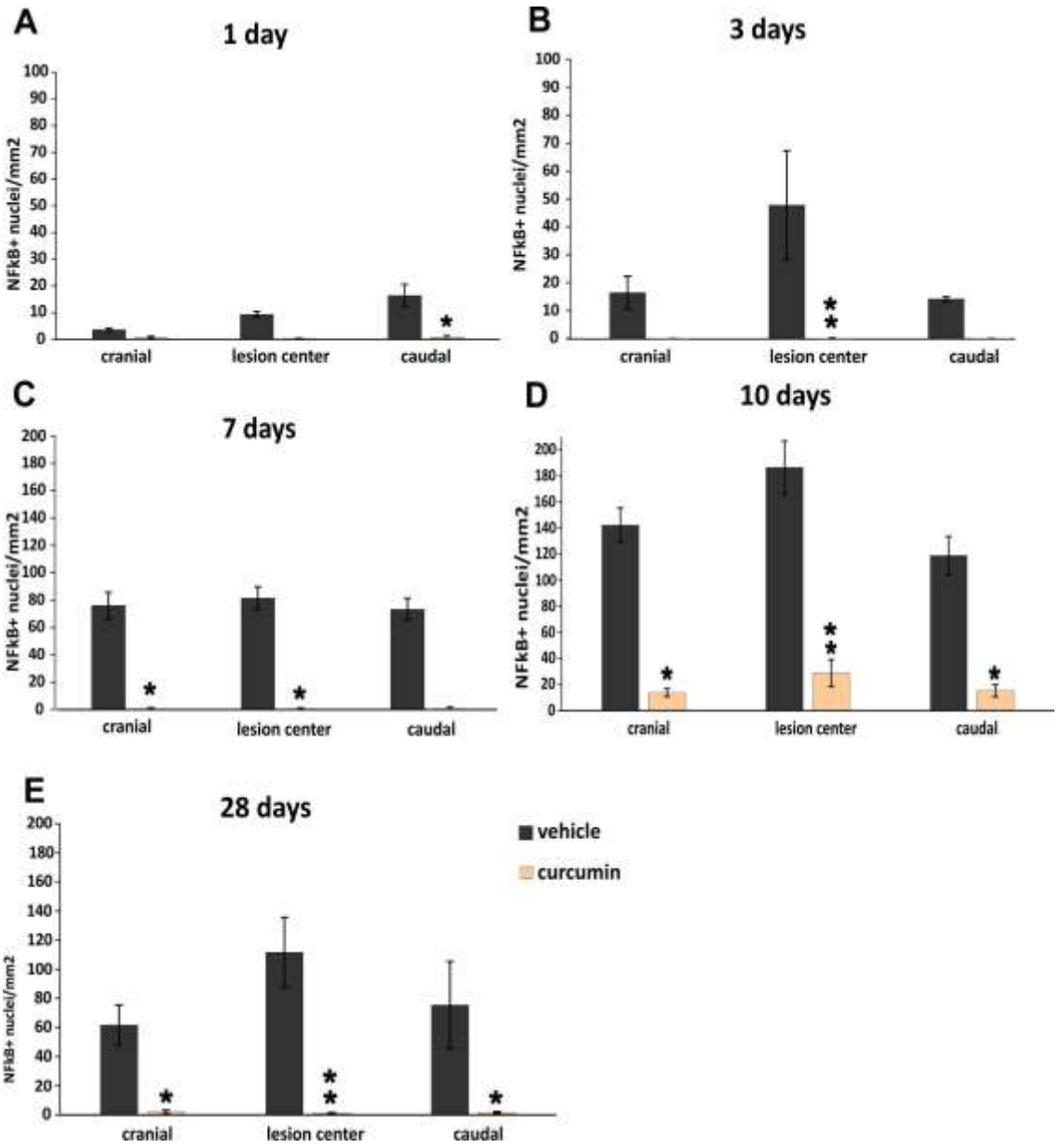

Fig.14 NFκB activity was assessed at days 1, 3, 7, 10 and 28 after SCI in control and curcumin treated rats. Graphs depict a significant decrease in NFκB activity measured by its nuclear translocation, in the central, cranial and caudal parts of the spinal cord lesion after curcumin treatment, which was observed at all time points. Data is expressed as mean and SEM with *$p < 0.05$, **$p < 0.01$ indicating statistical significance.

4.2.2. Cytokine production

Levels of secreted cytokines were determined using a customized Milliplex inflammatory cytokine kit (Millipore, Billerica, MA, USA) and Magpix instrumentation software MILLIPLEX (Millipore, Billerica, MA, USA). In animals treated with EGCG or their respective controls, cytokine production was assessed at 1, 3, 7, 10 and 14 days after induction of spinal cord injury, whereas in animals treated with curcumin or

vehicle, additional time point of 28 days after SCI was added. Both EGCG and curcumin proved to be potent modulators of cytokine production.

Treatment with EGCG was observed to be effective the most on the first day after SCI, with significantly reduced levels of MIP-α, IL-6 and RANTES (Fig. 15A), while levels in control group remained at stable elevated levels for 7 days and later decreasing to similar levels as in EGCG treated animals. Production of IL-4 showed significant increase in the EGCG treated group at day 1 compared to the saline treatment and continued to display insignificantly elevated levels throughout the experiment. We observed that the overall inflammatory response after EGCG treatment was decreased. Additionally, levels of IL-2 were significantly reduced 3 days after SCI (Fig. 15B) in the EGCG treated group and levels of IL-12 p70 and TNF-α gradually decreased during the first week of treatment after significant upregulation on the first day after SCI (Fig. 15A, B, C). Levels of IL-1b were significantly higher in the EGCG treated animals 14 days after SCI compared to controls (Fig 15E).

The most pronounced effect of curcumin treatment on cytokine production was observed in levels of MIP-1α, IL-2, IL-6, IL-12 p70, and RANTES and this was measured during the whole course of the experiment up until 28 days after SCI (Fig. 16). Additionally, changes in production rate of IL-4, IL-1β and TNF-α were detected in all time points. In curcumin treated rats, levels of IL-2 were found to be significantly lower than in untreated animals on the 1st and 3rd day after SCI and then again 14 and 28 days after injury (Fig. 16A, B, E, F). On days 7 and 10, IL-2 levels still tend to be lower, although without reaching statistical significance (Fig. 16C, D). Levels of TNF-α were significantly lowered in the spinal cords of animals receiving curcumin compared to controls 1 and 14 days after SCI (Fig 16A, E). In contrast, RANTES and MIP-1α were detected in higher concentrations in control spinal cords at the beginning and at the end of the 28-day period. Animals treated with curcumin produced significantly higher levels of IL-6 and IL-12 p70 than vehicle treated controls 14 and 28 days after SCI (Fig. 16E, F). Curcumin treated group showed significantly higher levels of IL-4 on the 28th day following SCI (Fig. 16F).

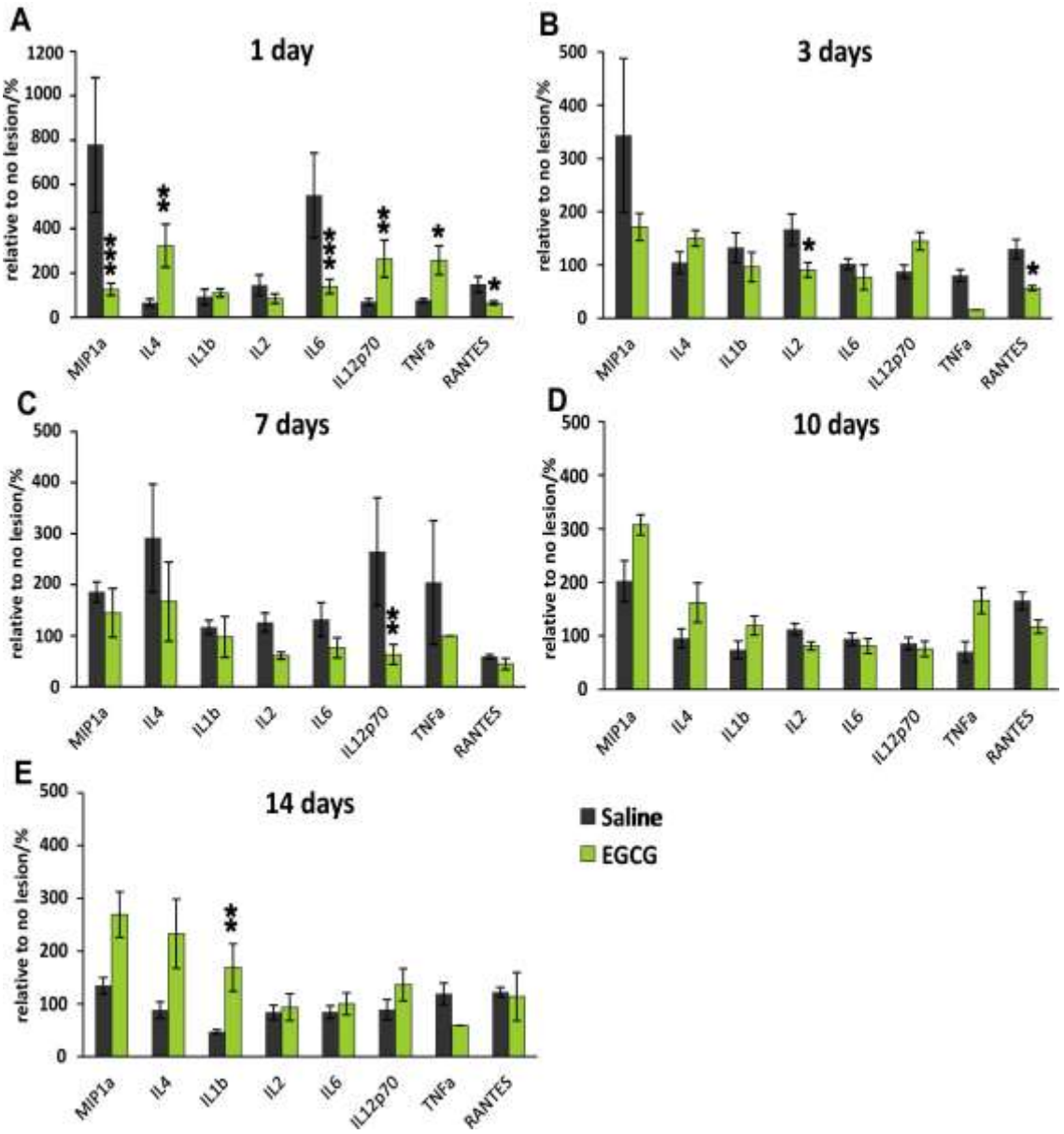

Fig. 15 Levels of pro-inflammatory cytokines 1, 3, 7, 10 and 14 days after SCI in EGCG and saline-treated groups. Levels of MIP-1α, IL-6 and RANTES were downregulated after EGCG treatment compared to saline treatment 1 day after SCI **(A)**. Levels of IL-1b (14 days), IL-4 and TNF-α (1 day) were significantly upregulated in EGCG-treated group **(A, E)**. The first day after SCI, IL-12 p70 was upregulated **(A)** after EGCG treatment, but 7 days after SCI **(C)** it was significantly reduced compared to controls. Data is expressed as mean and SEM with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ indicating statistical significance.

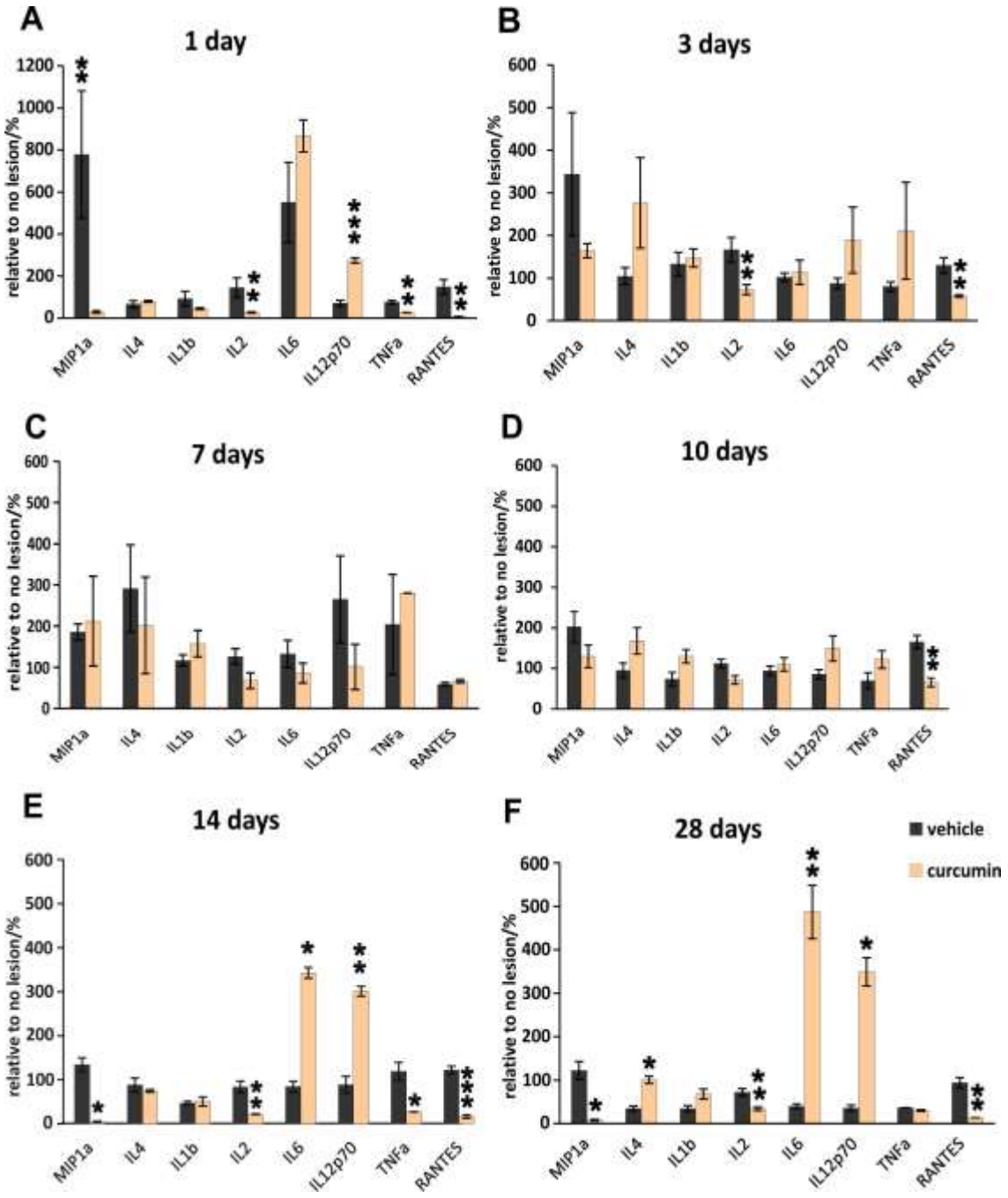

Fig. 16 Levels of pro-inflammatory cytokines 1, 3, 7, 10, 14, and 28 days after SCI in curcumin- and vehicle-treated rats. The levels of IL-2 were significantly lower in the curcumin-treated group compared to vehicle treatment 1 and 3 days after SCI (**A**, **B**); The levels of cytokines 7 and 10 days after SCI were not significantly influenced by curcumin treatment with the exception of RANTES, which was more abundant in control spinal cords 1, 3, 10, 14, and 28 days after SCI (**C**, **D**); The levels of TNF-α reached statistical significance in curcumin-treated spinal cords compared to controls 1 and 14 days after induction of the lesion. IL-2 again significantly decreased 14 and 28 days after injury in the curcumin group (**E**, **F**). IL-6 and IL-12p 70 were upregulated by curcumin treatment on day 1 and then 14 and 28 days post-lesion. MIP-1α reached significantly higher levels in the control group 1, 14 and 28 days after SCI. Data is expressed as mean and SEM with * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ indicating statistical significance.

4.2.3. Gene expression

At 10 and 28 days after spinal cord injury, using qRT-PRC, we determined the expression of genes involved in regeneration processes such as axonal sprouting (*Gap43*), growth factor production (*Vegfa, Sort1, Fgf2, Cntf*), glial cells response (*Gfap, Olig2*) as well as inflammatory response, namely polarization of macrophage phenotypes (*Cd86, Irf5, Cd163, Mrc1*), and expression of *Nfkb1*, which encodes p105 later processed to produce p50, one of Rel proteins capable of forming NFκB dimers. Typical dimer involved in canonical pathway consists of p50 and p65.

EGCG treatment caused upregulation of *Fgf2* and *Vegf* genes in the spinal cord tissue 28 days after SCI (Fig. 17B). Macrophage phenotype related genes were modulated by EGCG treatment, significantly elevating all genes (*Cd86, Irf5, Cd163, Mrc1*) when compared to saline treated animals. *CD86* gene showed a trend toward statistical significance in its reduction over time suggesting decreasing number of $CD86^{+}$ macrophages at injury site. On the contrary, *CD163* gene displayed a strong trend toward statistical significance in its increase over time (Fig. 17C, D).

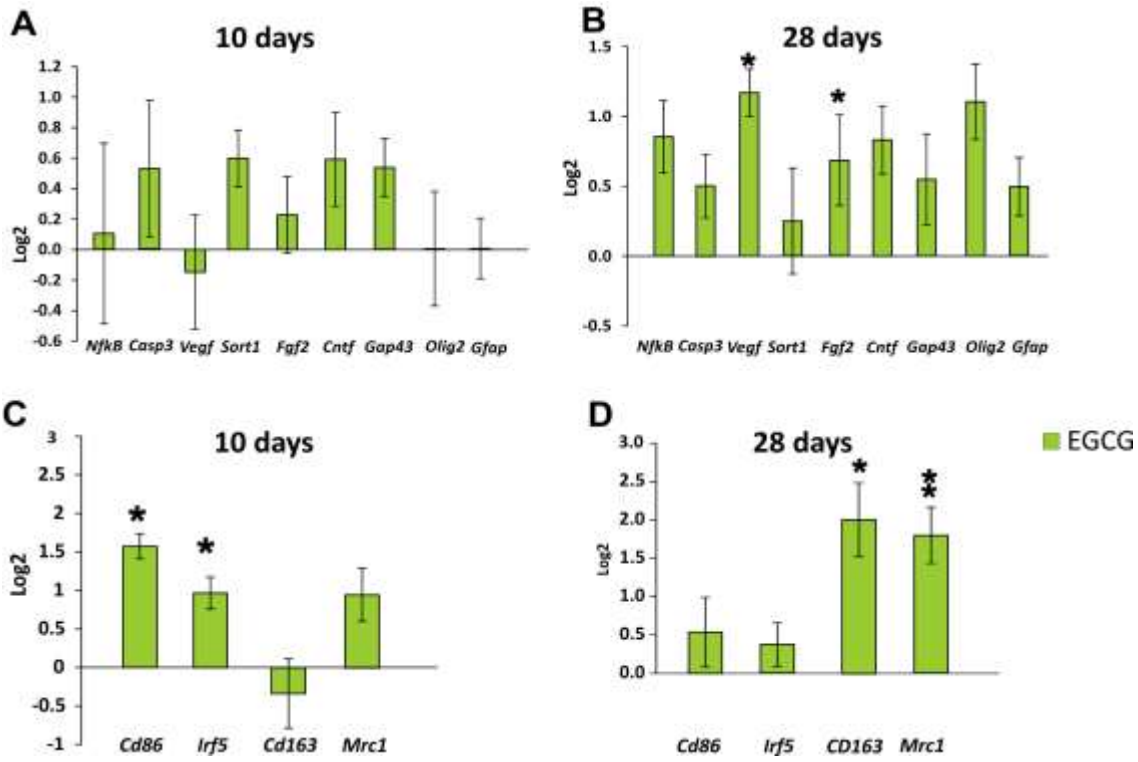

Fig. 17 Gene expression reflecting regenerative processes in the centers of spinal cord lesions, 10 and 28 days after SCI relative to saline (**A, B**). Genes related to the macrophage phenotypes M1/M2 (**C, D**). Data is expressed as mean and SEM (ΔΔCt values of EGCG vs. saline) with * $p < 0.05$, ** $p < 0.01$ indicating statistical significance.

Expression of studied genes was not markedly changed after curcumin treatment. Significant decrease of mRNA transcription was detected in case of *Gfap* at 10 days, while *Irf5* expression was upregulated at 10 days and *Gap43* at 28 days (Fig. 18A, B). This decrease of expression of *Gfap* was in line with our histological findings, where a significantly smaller $GFAP^+$ area was found in animals treated with curcumin (Fig. 22). Similarly, the downregulation of *Rantes* and *NFkB1* levels corresponded with results of the cytokine analysis and, in case of NFκB1, also to the immunohistochemical staining of NFκB $p65^+$ nuclei suggested inhibition of canonical NFκB pathway.

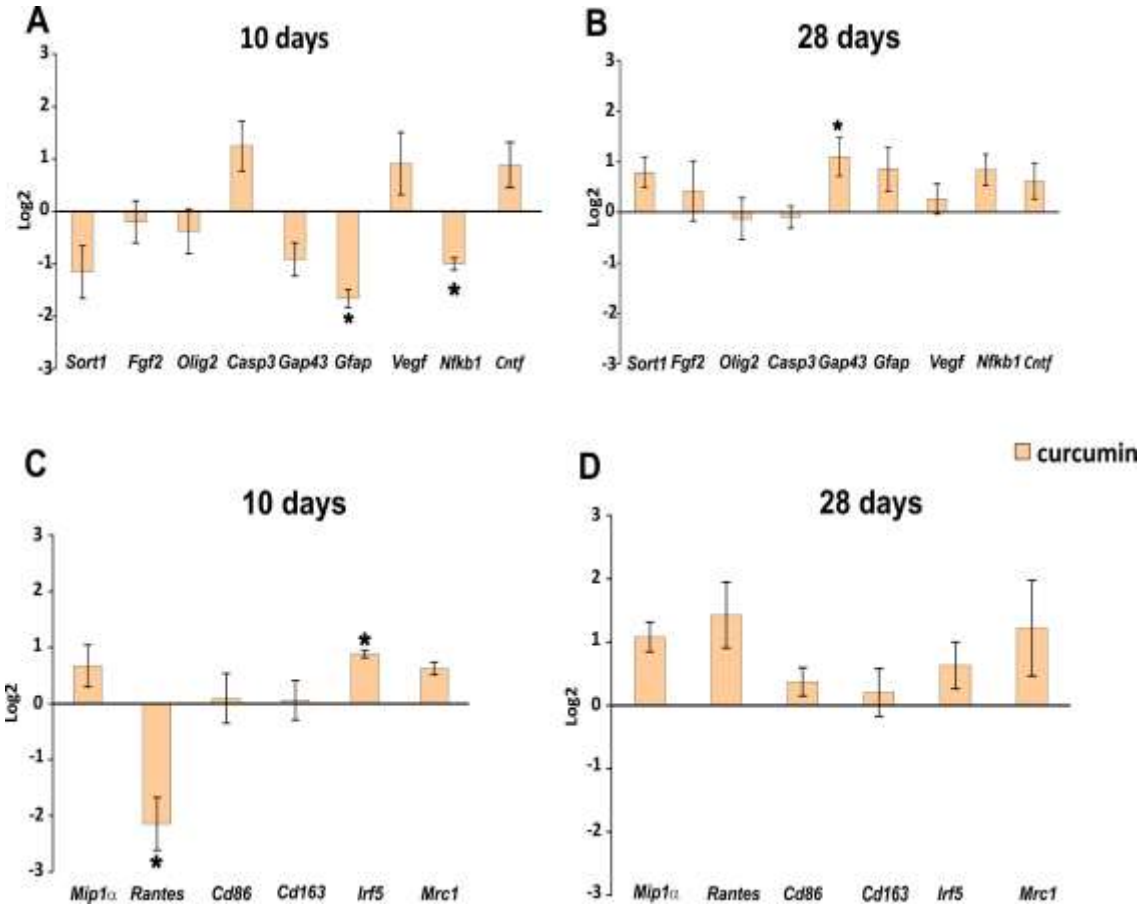

Fig. 18 Gene expression in lesion centers 10 and 28 days following SCI. Graphs show genes related to regenerative processes (growth factors, apoptosis, axonal sprouting, astrogliosis) (**A**,**B**) and to immune response (cytokines macrophage phenotypes M1/M2) (**C**,**D**).Graphs show the log2-fold changes of the indicated genes 10 and 28 days after curcumin treatment in comparison to the control values. Data is expressed as mean and SEM (ΔΔCt values of curcumin vs. vehicle) with * $p < 0.05$ indicating statistical significance.

4.2.4. Sparing of white and gray matter and changes in cavity size

Morphometric measurement or gray and white matters was performed to determine levels of preservation of the spinal cord tissue after treatments with anti-inflammatory

compounds or their respective controls. This data was also used to calculate cavity sizes 9 weeks after spinal cord injury.

After treatment with EGCG, most of the preservation of white matter in spinal cords was detected cranially to the lesion center when compared to saline controls. However, this observation did not reach statistical significance (Fig 19A). The EGCG treatment resulted in gray matter sparing in both caudal and cranial areas of the spinal lesion when compared to controls and reached significance 5 mm caudally from the lesion center (Fig. 19B). Calculated volume of lesion cavities was similar in both treatment and control groups with observed statistical significance.

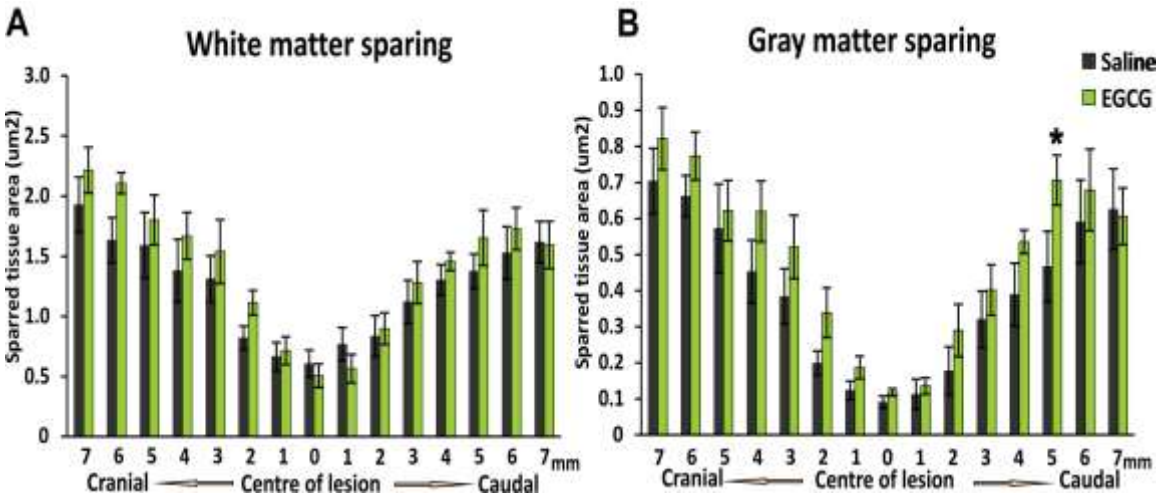

Fig. 19 Area of spared white matter **(A)** and gray matter **(B)** after treatment with EGCG or saline in lesion centers and in cranial and caudal areas of spinal cords. Data is expressed as mean and SEM with * $p < 0.05$ indicating statistical significance.

After curcumin treatment, insignificantly larger portion of white matter was preserved when compared with control group, which was observed at 4–5 mm caudally and 5–7 mm cranially from the lesion center. Gray matter in the curcumin treated group was also more preserved 4–5 mm caudally and 4–7 mm cranially from the spinal cord lesion when compared to controls, but this was not shown to be significant (Fig. 20). Volume of cavities in the spinal cord tissue was significantly smaller after curcumin treatment compared to controls, while no difference in cavity size was found after EGCG treatment Fig. 21).

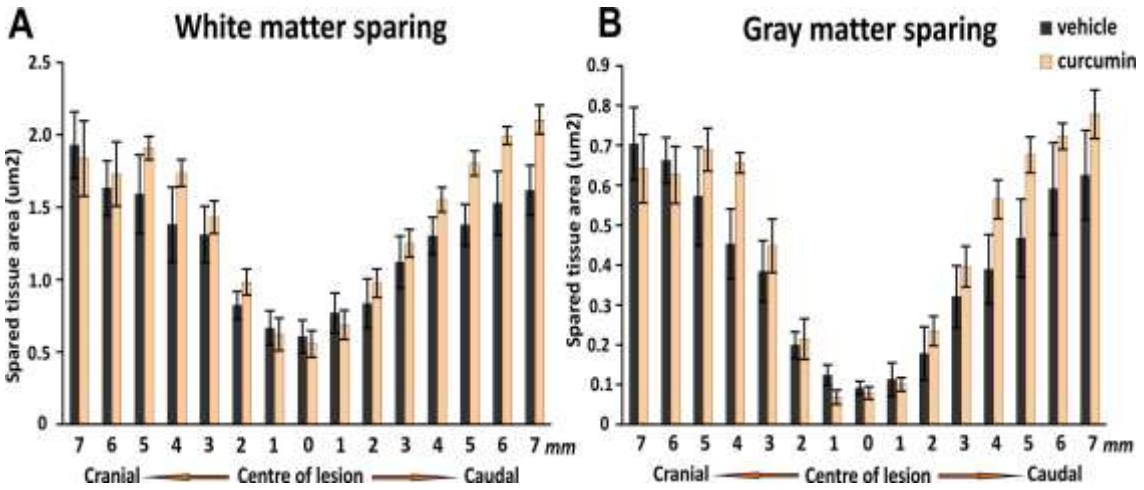

Fig. 20 Area of spared white **(A)** and gray matter **(B)** after treatment with curcumin or vehicle in lesion centers and in cranial and caudal areas. Data is expressed as mean and SEM with * $p < 0.05$ indicating statistical significance.

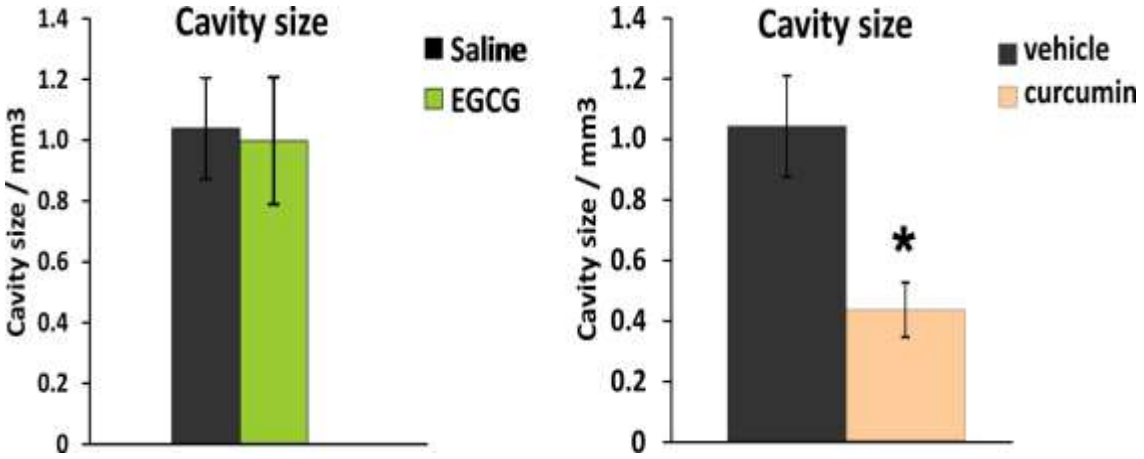

Fig. 21 Cavity size of saline treated animals compared to animals treated with EGCG **(A)** or curcumin **(B)**. Data is expressed as mean and SEM with * $p < 0.05$ indicating statistical significance.

4.2.5. Glial scar and astrocyte activation

Area positive to GFAP staining was measured 9 weeks after injury in animals as well as the number of protoplasmic astrocytes. Glial scar formation was compared between animals treated with EGCG or curcumin and their respective controls. In animals treated with EGCG, the area of glial scar was larger in saline treated rats, although this observation did not reach statistical significance (Fig. 22A). The number of protoplasmic astrocytes was also higher in saline treated rats, which reached statistical significance at 3 mm cranially from the lesion center (Fig. 22B).

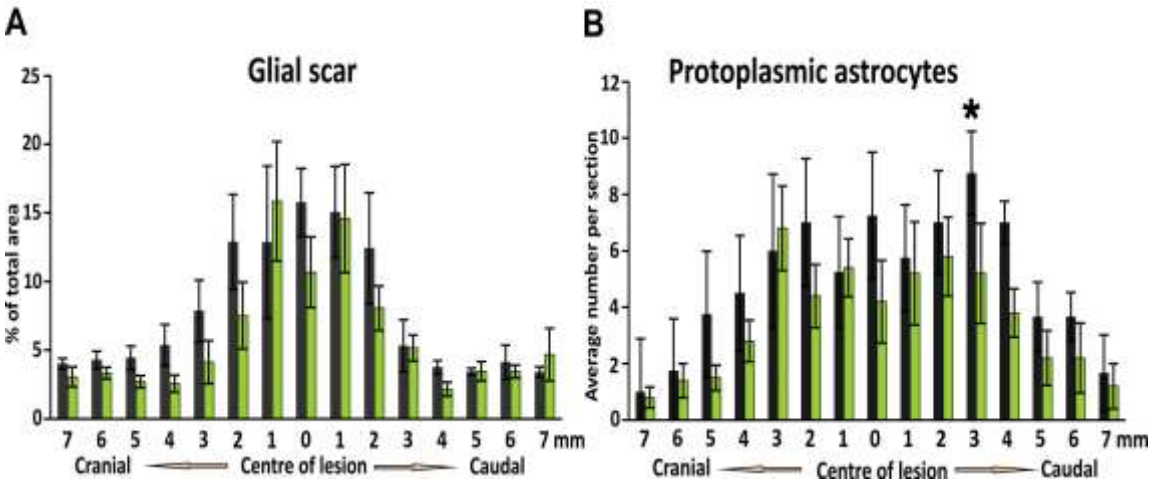

Fig. 22 Total GFAP positive area in cranial, central and caudal areas of spinal cord lesion depicting glial scar distribution **(A)** and average number of protoplasmic astrocytes in all 15 sections of spinal cords **(B)** of animals treated with EGCG or saline. Data is expressed as mean and SEM with * $p < 0.05$ indicating statistical significance.

Distribution of glial scar in curcumin treated rats was similar with controls in peripheral regions of spinal cord, however significantly stronger positivity was seen around the central part of the lesion area in control animals suggesting its attenuation due to curcumin treatment (Fig. 23A). Moreover, the number of protoplasmic astrocytes was significantly higher in lesion center and its vicinity in animals of the control group (Fig. 23B).

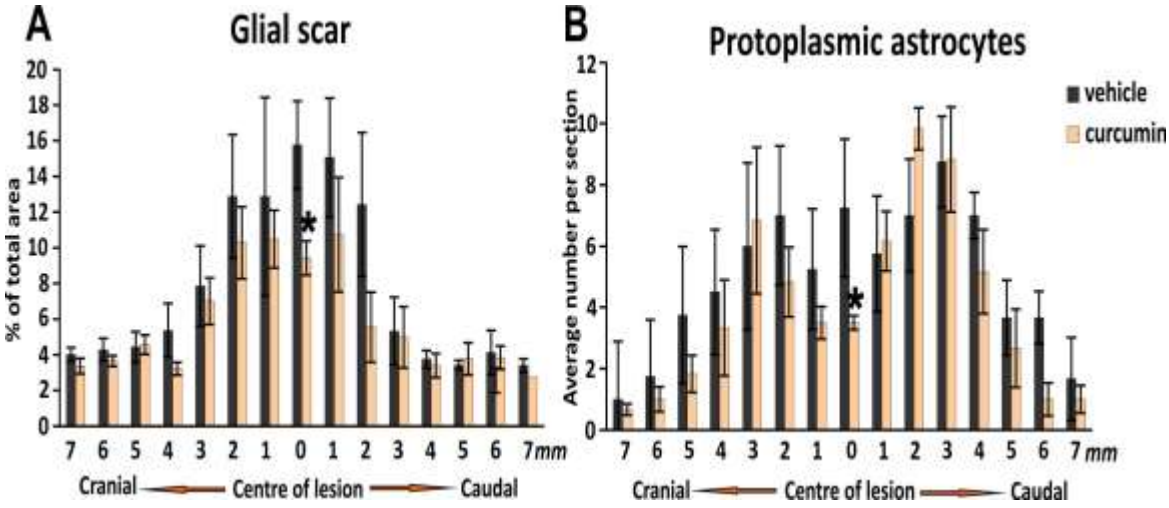

Fig. 23 Total GFAP positive area in cranial, central and caudal areas of spinal cord lesion depicting glial scar distribution **(A)** and average number of protoplasmic astrocytes in all 15 sections of spinal cords **(B)** of animals treated with curcumin or vehicle. Data is expressed as mean and SEM with * $p < 0.05$ indicating statistical significance.

4.2.6. Axonal sprouting

The level of axonal sprouting was assessed by counting GAP43 positive fibers 9 weeks after SCI. Analysis of GAP43^{+} fibers after EGCG treatment showed a significantly enhanced level of axonal sprouting compared to controls (Fig. 24A, B). Despite EGCG proving to be a simulator of new axonal growth, treatment with anti-inflammatory substance curcumin did not prove to have such effect (Fig. 25).

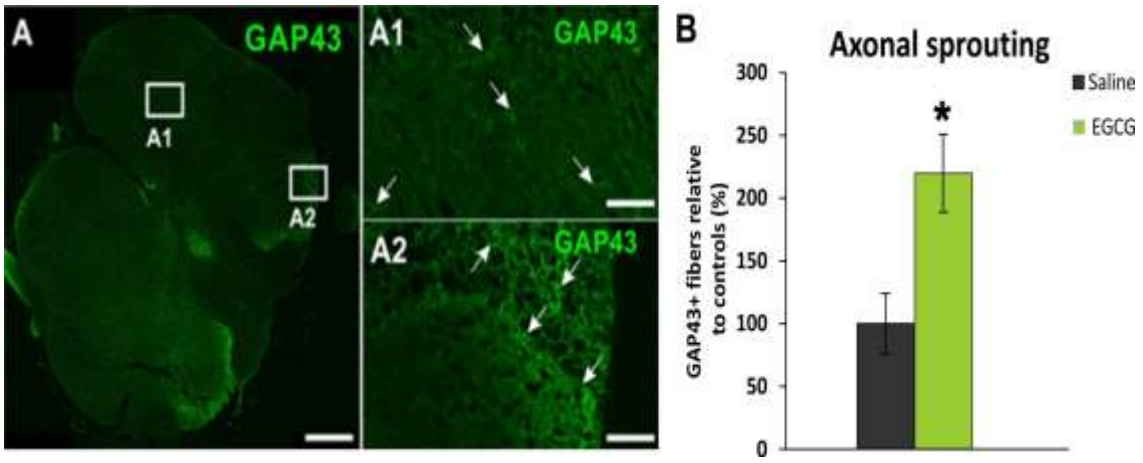

Fig. 24 Expression of GAP43 on transversal section of the spinal cord lesion after EGCG treatment. Arrows indicate GAP43 positive newly sprouted axons. Scale bar 500 µm **(A)**, 20 µm **(A1)**. EGCG treatment significantly supports axonal sprouting in spinal cord lesion **(B)**. Data is expressed as mean and SEM with * $p < 0.05$ indicating statistical significance.

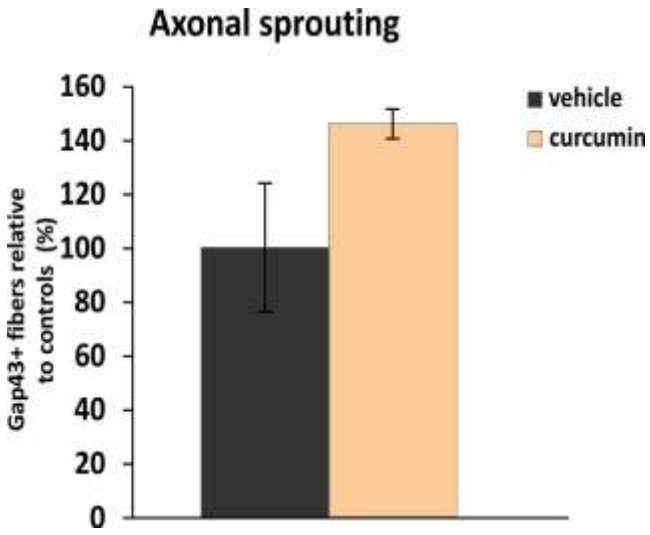

Fig. 25 Amount of GAP43^{+} fibers in spinal cords of rats treated with curcumin or vehicle. Significant differences were not found. Data is expressed as mean and SEM.

4.2.7. Functional outcomes

A set of behavioral tests was used to determine changes in motor and sensory functions in hind limbs of rats treated with anti-inflammatory compounds or control animals. Locomotive function of hind limbs was determined once per week for 9 weeks after SCI using the BBB motor performance test, flat beam test and rotarod test. At day 0 after inducing spinal cord compression lesion, rats were scored with 0, representing no movement and control of hind limbs. A withdrawal latency of hind limbs to thermal stimulus was evaluated once per week to detect changes in sensory function using Plantar test.

Flat beam test was used to measure recovery of motor function and coordination of rat hind limbs. Flat beam test reveals the level of coordination and balance. This test precisely detects motor deficits and their improvement in time. Immediately after SCI, performance of rats treated with EGCG started to improve, when compared to saline-treated animals, which became significant in the 6th week of treatment (Fig. 26A). Significant improvement in their ability to cross the beam was also detected in shorter to time needed to transverse the beam (Fig. 26B). In the first 4 weeks, rats regained their ability to move utilizing all three joints in their hind limbs, however no significant difference was detected after treatment with EGCG when compared to the saline controls in the BBB test. After the 5th week, rats continued to recover slightly. However, overall significance of EGCG treatment was not detected (Fig. 26C). No significant difference between saline and EGCG treated rats was detected in rotarod test (Fig. 26E). Administering EGCG resulted in slightly decreased withdrawal latency in Plantar test up until the 4th week of treatment, although this was not significantly different from control animals. After the 4th week, withdrawal times were stabilized and no significant differences were observed between the two groups. Slight hyperalgesia (thermal nociception), resulting from SCI was not affected by EGCG treatment (Fig. 26D).

Treatment with curcumin proved to significantly enhance locomotor recovery, particularly in the first week. Two and three weeks after SCI and start of curcumin treatment, rats continued to display significantly better locomotor performance than vehicle treated rats in open field test. In the later weeks, performance of rats from the curcumin group was better and significantly at week 7, however performances in other weeks were insignificant (Fig. 27A). Similarly to EGCG, no significant differences

from control animals were detected after treatment with curcumin after Plantar test. However, insignificant increase of withdrawal latency was observed in curcumin treated group 5 and 6 weeks after SCI (Fig. 27B).

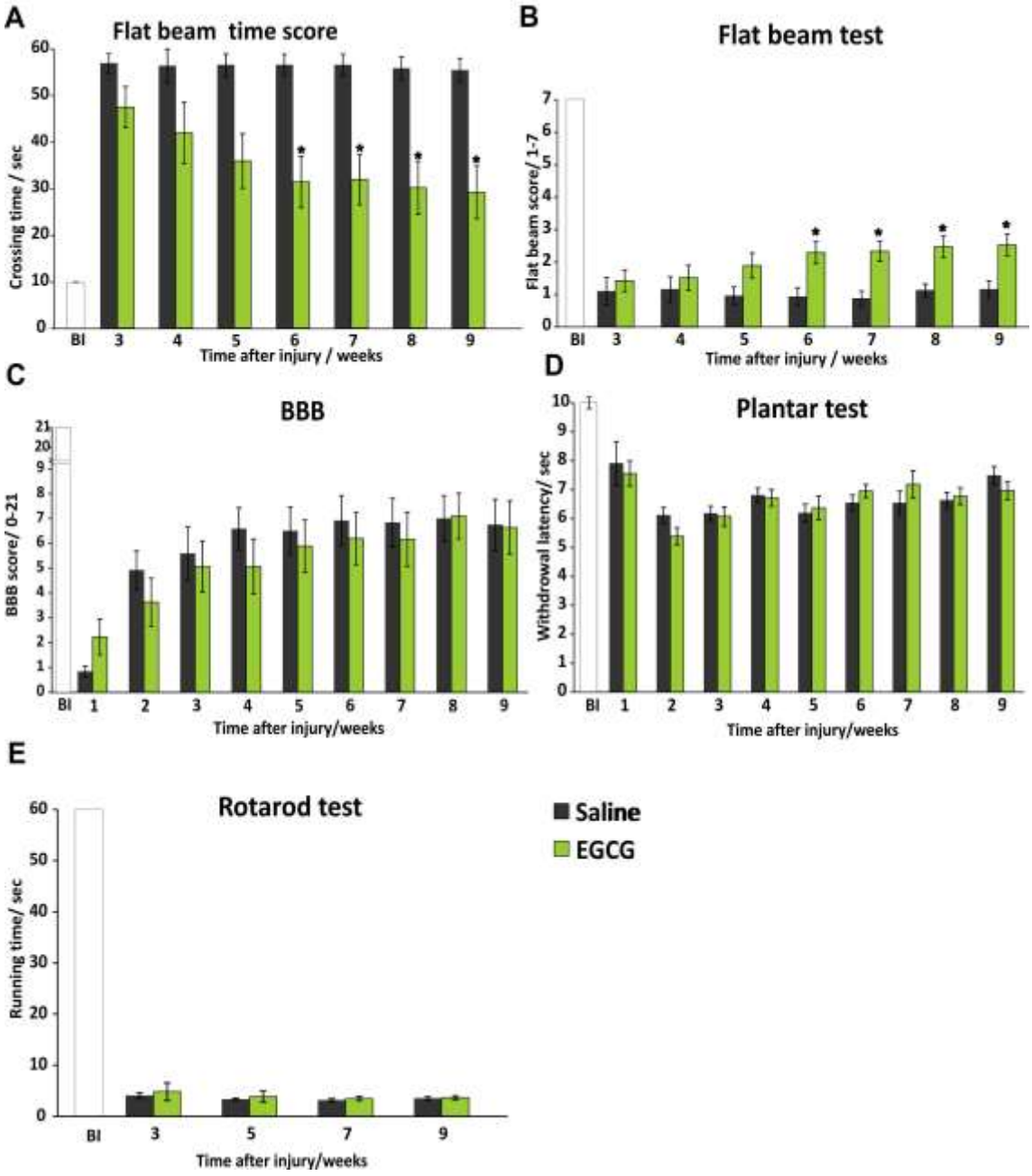

Fig. 26 Behavioral performance of rats after saline and EGCG treatments. Flat beam test used to determine complex motor performance of rats showed improvement after EGCG treatment when compared to saline treatment **(A, B)**. Open field BBB test showed no significant difference between saline and EGCG treatments **(C)**. Thermal sensitivity measured by the Plantar test reached the same withdrawal latency in both groups **(D)**. Rotarod test showed that the ability to balance on the rotating pole remained at the same level during the entire testing period in saline and EGCG groups **(E)**. Data is expressed as mean and SEM with $*p < 0.05$ indicates statistical significance, BI-before injury.

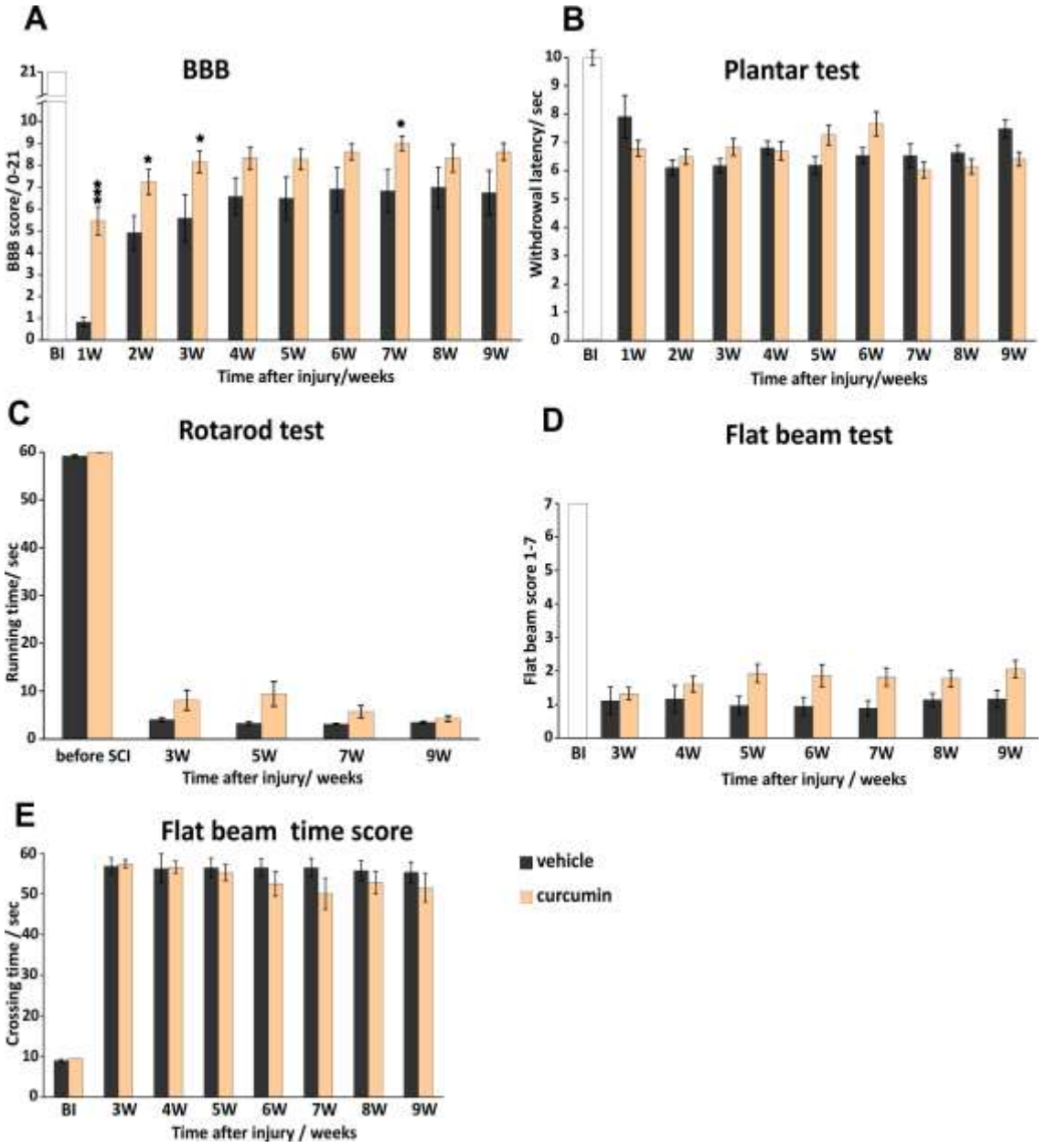

Fig. 27 Changes in motor and sensory functions of rats treated with curcumin or vehicle. BBB open field test revealed strong improvement curcumin-treated animals. Statistically significant differences were observed 1, 2, 3, and 7 weeks after curcumin treatment **(A)** Sensory Plantar test revealed no significant effect on hyperalgesia after curcumin treatment **(B)**. Rotarod device tested the ability of the rats to balance on a rotating pole and did not reveal statistically significant changes (**C**). Flat beam test was used to evaluate muscle strength and forelimb-hind limb coordination in both the control and curcumin groups. Curcumin treated group performed better on the flat beam test throughout the whole study period, but differences were not statistically significant **(D)**. Flat beam time score reflects the ability of the animals to cross the beam within a maximum of 60 s, no significant differences between the groups were observed (**E**). Data is expressed as mean and SEM with * $p < 0.05$, and *** $p < 0.001$ indicating statistical significance. BI-before injury.

4.3. Effects of stem cells treatments on spinal cord injury

The third section of results is based on results from listed articles:

Ruzicka J, Machova-Urdzikova L, Gillick J, Amemori T, Romanyuk N, Karova K, Zaviskova K, Dubisova J, Kubinova S, Murali R, Sykova E, Jhanwar-Uniyal M, Jendelova P. (2017) A Comparative Study of Three Different Types of Stem Cells for Treatment of Rat Spinal Cord Injury. Cell Transplant. Apr 13;26(4):585-603.

Urdzíková LM, Růžička J, LaBagnara M, Kárová K, Kubinová Š, Jiráková K, Murali R, Syková E, Jhanwar-Uniyal M, Jendelová P. (2014) Human mesenchymal stem cells modulate inflammatory cytokines after spinal cord injury in rat. Int J Mol Sci. Jun 25;15(7):11275-93.

4.3.1. Survival and differentiation of implanted stem cells

Survival of IT (MSCs) and IS (SPC-01 and iPSC-NPs) implanted stem cells was evaluated 2 weeks and 2 months after transplantation (TX), respectively. Only a few MSCs were observed to be partially attached to the dorsal spinal cord surface and none were detected 2 weeks after grafting (Fig. 28A). In contrast, higher numbers of neural precursors derived from either SPC-01-GFP cells (16 ± 3%) or iPS cells (11 ± 3%) were detected around the injection site 2 months after grafting (Fig. 28B, C). Both types of human neural precursors remained rather immature as revealed by staining 2 months after implantation. SPC-01-GPF cells were predominantly $GFAP^+$ or expressed early neural markers such as NESTIN, OLIG2, and NKX6-1 (Fig. 28 D-F). Most of the iPSC-NPs were positive for neural βIII-TUBULIN, MAP2, and DCX, with some of the grafted cells being $GFAP^+$ (Fig. 28 G-J).

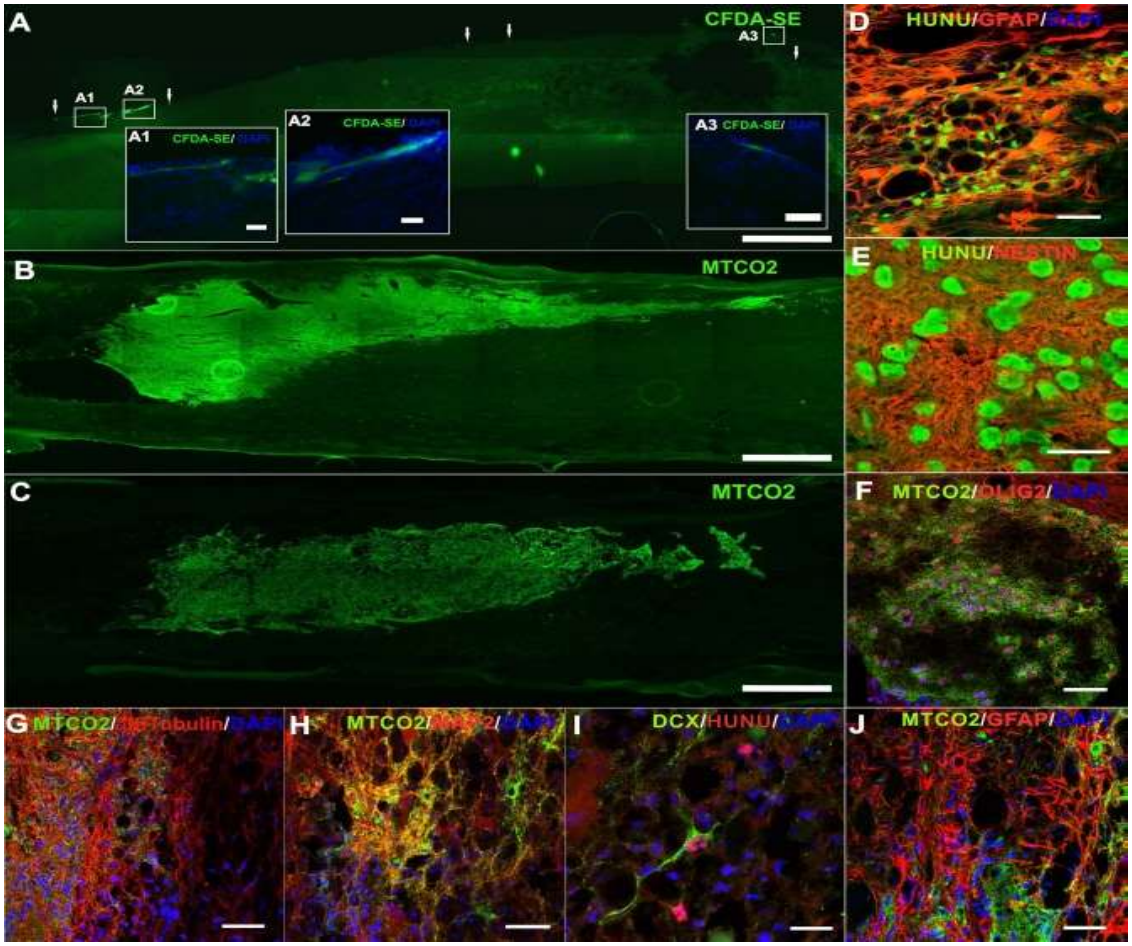

Fig. 28 Survival of implanted stem cells and differentiation of SPC-01 and iPSC-NP cells following their implantation after SCI. Only a few MSCs (CFDA-SE) were detected on the spinal cord surface 2 weeks after application **(A)**, while SPC-01 cells **(B)** and iPSC-NPs **(C)** (MTCO2) survived robustly for at least 2 months after implantation. Arrows mark the attached MSCs. Inserts **(A1-A3)** show MSC morphology. Differentiation of SPCs **(D-F)** and iPSC-NPs **(G-J)** demonstrated that the majority of SPCs differentiated into astrocytes **(D)** or remained immature **(E, F)**, while transplanted iPSC-NPs expressed neuronal markers **(G-I)** and also astrocytic GFAP **(J)**. **(D), (F)-(H)**, and **(J)** photos were taken on the edge of the graft, while **(E)** is from the center of grafts. **(I)** shows cell migration into the host tissue. Scale bars: 300 μm **(A)**, 500 μm **(B, C)**, 20 μm **(A1, A2, A3, D-J)**.

4.3.2. Modulation of NFκB activity after cell transplantation

Analysis of p65 nuclear translocation revealed elevation of its activity across groups in the first two weeks of experiments. NFκB activity significantly increases in the lesion center of control animals receiving saline only (Fig. 29A, Fig. 30B, B1), but also in animals transplanted with iPS-NPs (Fig. 29C). These two groups displayed very similar pettern of p65 nuclear translocation suggesting little effect of iPS-NPs on NFκB activity. Contrary to this are findings in both SPC-01 and MSCs groups. Both types of cells strongly decreased NFκB activity in lesion centers of spinal cords analyzed 28

days after inducing injury. Spinal neural precursors inhibited p65 translocation more strongly than MSCs, but both decreased NFκB by about two thirds (Fig. 31, Fig. 30C)).

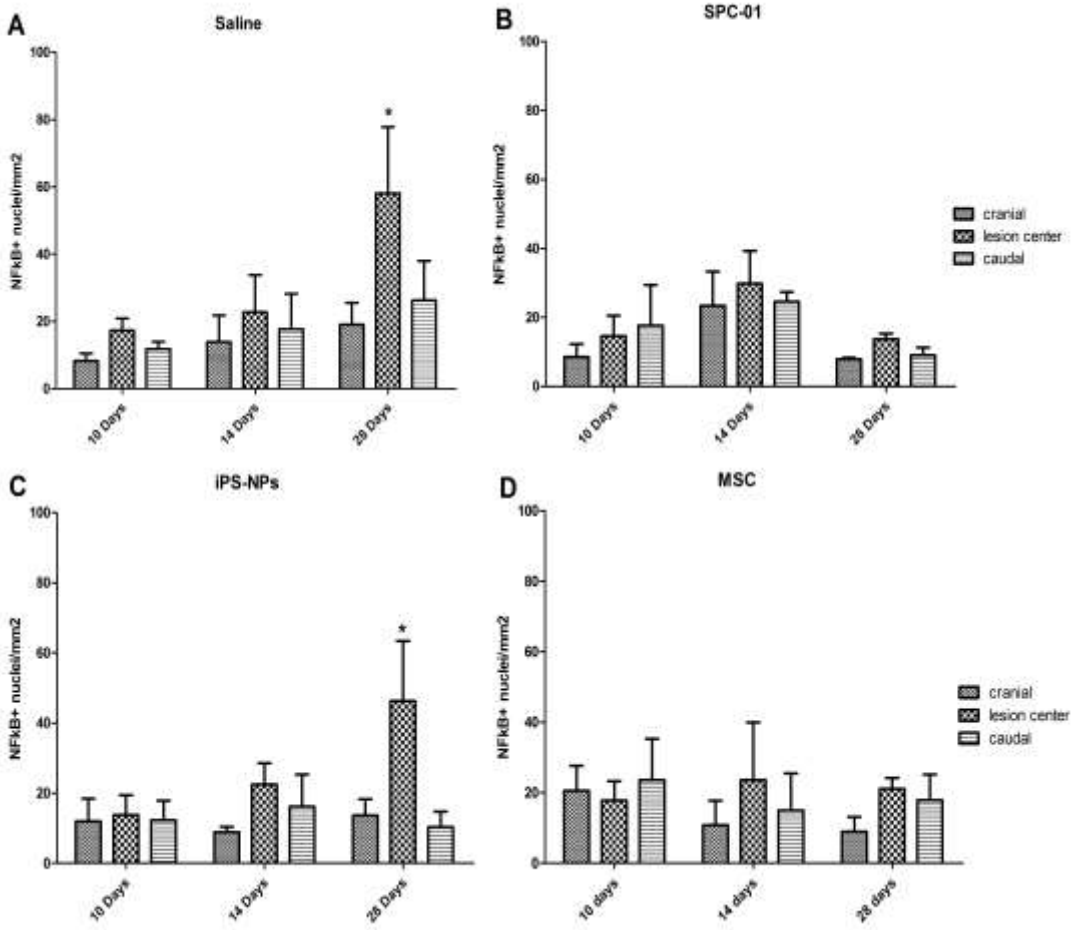

Fig. 29 Density of NFκB^{+} nuclei 10, 14 and 28 days after SCI/3, 7, 21 days after implantation of saline **(A)**, SPC-01 **(B)**, iPS-NPs **(C)** or MSCs **(D)**, in lesion centers and cranially and caudally from injury/transplantation sites. Data is expressed as mean and the standard error of the mean (SEM) with $*p < 0.05$, indicating statistical significance.

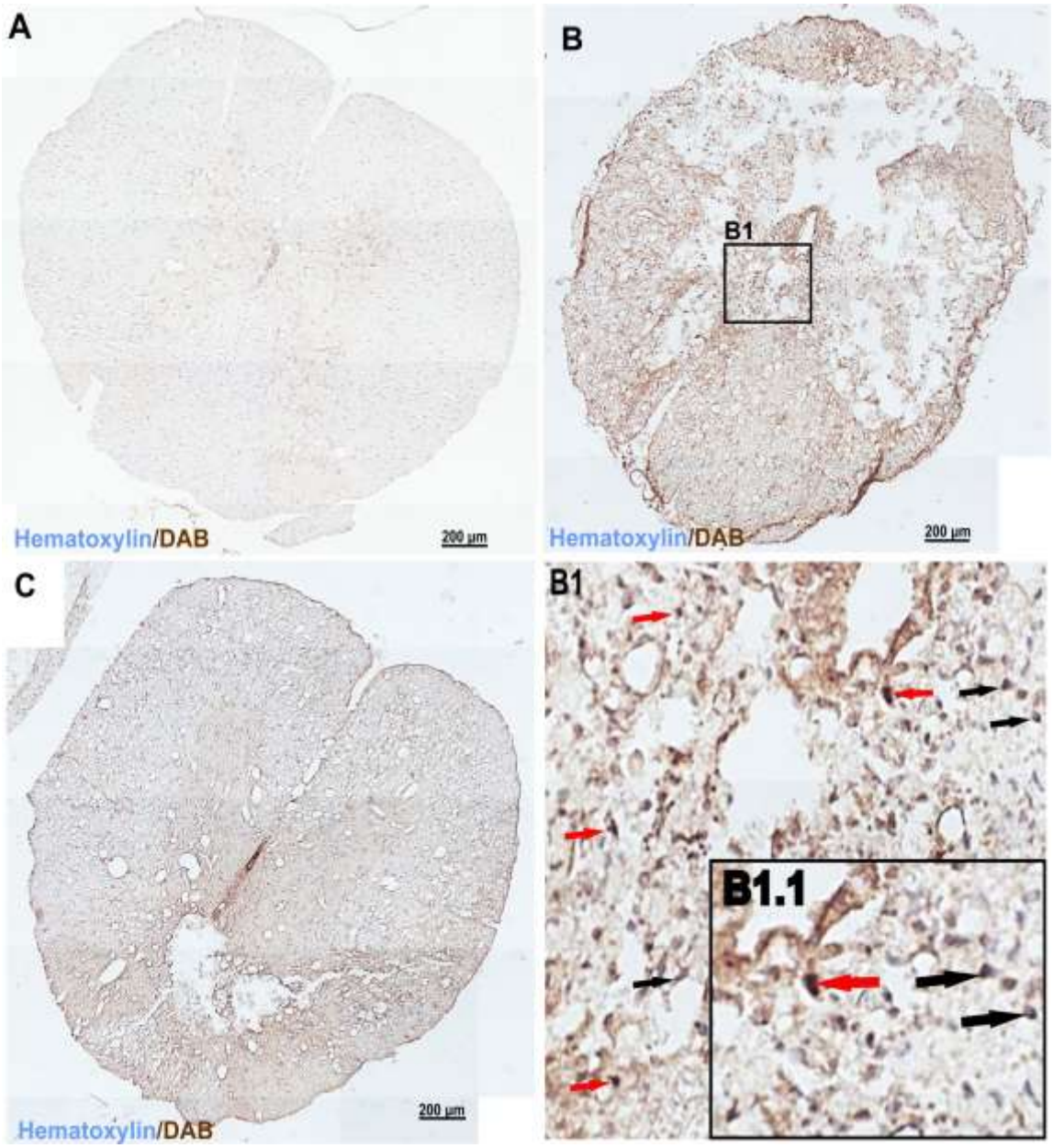

Fig. 30 Representative images show cross sections of the spinal cords stained with hematoxyline and NFκB-DAB in healthy animals **(A)**, 28 days after SCI + saline treatment **(B)**, 28 days after SCI + SPC-01 treament **(C)**. Red arrows indicate nuclei with translocated p65 proteins of the NFκB dimer and black arrows indicate $NF\kappa B^-$ nuclei stained with hematoxyline **(B1, B1.1)**.

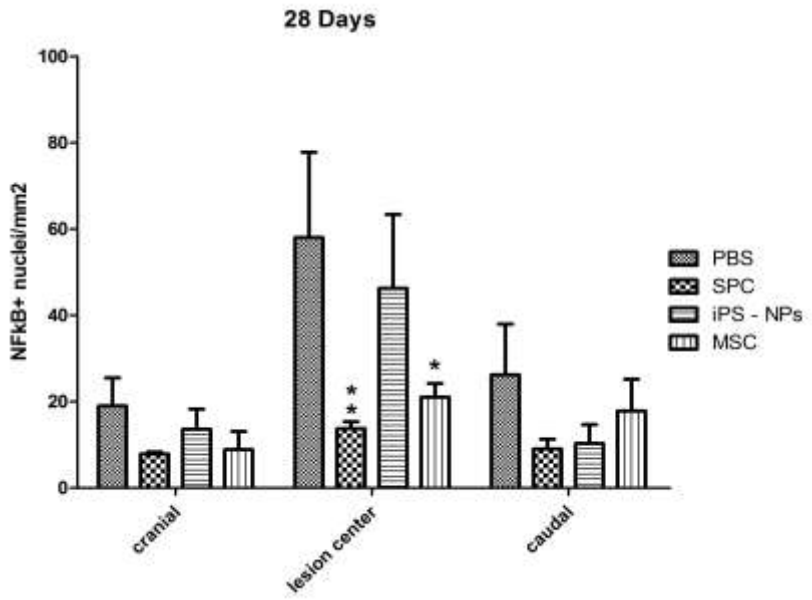

Fig. 31 NFκB activity in spinal cords of control or transplanted animals 28 days after SCI/21 days after transplantation in all 3 studied spinal cord locations. Data is expressed as mean and the standard error of the mean (SEM) with *$p < 0.05$, **$p < 0.01$ indicating statistical significance.

4.3.3. Cytokine production

Inflammatory response to spinal cord injury and stem cells transplantation was defined by cytokine production measurement using a customized Milliplex kit (Millipore, Billerica, MA, USA) and Magpix instrumentation software MILLIPLEX (Millipore, Billerica, MA, USA). Levels of cytokines MIP-1α, IL-4, IL-1β, IL-2, IL-6, IL-12p70, TNF-α, and RANTES were determined at 10, 14, and 28 days after SCI or 3, 7 and 21 days after transplantation. In all three treatment groups, production levels of MIP-1α, IL-2, and TNF-α cytokines were decreased relative to those of saline treated controls at 10 days post-SCI and this persisted through day 14 (Fig. 32A, B). However, at day 28 in the MSC group, MIP-1α, IL-2, and TNF-α levels increased in comparison those of other treatment groups or saline treated group (Fig. 32C). The production of chemokine RANTES was significantly downregulated in all time points when compared to saline control levels in SPC-01-GFP and iPSC-NP groups, however it was upregulated in case of all MSC treated rats. IL-4 levels in neural precursor treated groups gradually increased over time, while MSC treated rats had decreased production of IL-4 at day 10, which increased insignificantly at day 28 (Fig. 32A, C). Levels of strong pro-inflammatory cytokine IL-1β were significantly lowered in animals transplanted with iPSC-NPs, but insignificantly in case of MSCs and SPC-01-GFP compared to saline 3 days after transplantation (Fig. 32A). At 14 and 28 days after SCI, levels of IL-1β

remained similar to those of the saline treated group, apart from an increase in the SPC-01-GFP group on day 14 after SCI (Fig. 32B, C). IL-6 and IL-12p70 levels were noticeably higher in both NP groups compared to control and MSC groups. This pattern continued throughout the whole experimental period (Fig. 32A, B, C).

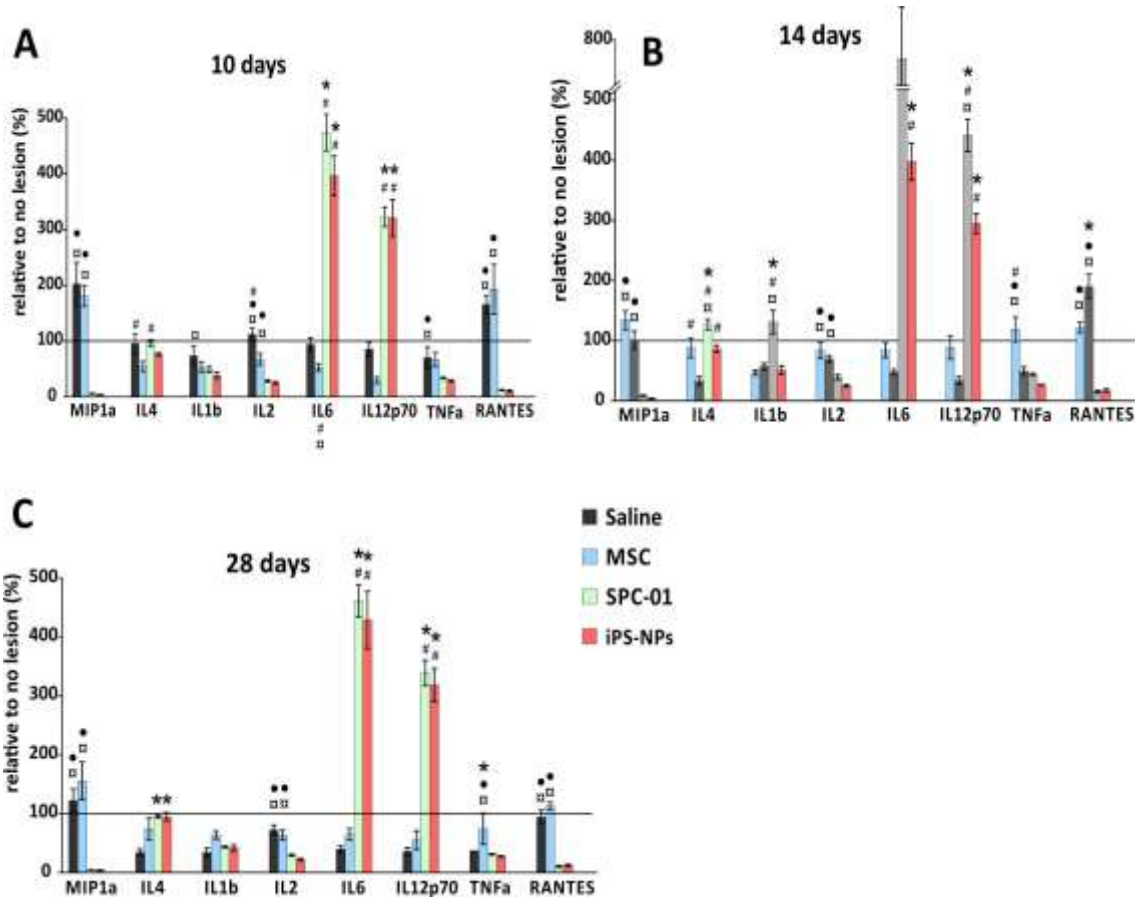

Fig. 32 Levels of cytokines and chemokines produced in spinal cords after stem cell treatments 10 **(A)**, 14 **(B)**, and 28 **(C)** days after SCI, which correspond to 3, 7, and 21 days after implantation. Levels measured in healthy animals were set to 100% indicated by a horizontal line in the graphs. Data is presented as mean and SEM with $^{*}p < 0.05$ versus saline; $\#p < 0.05$ versus MSCs; $\bullet p < 0.05$ versus SPC-01; $¤p < 0.05$ versus iPSC-NPs.

4.3.4. Gene expression

The relative expression of rat genes related to growth factors [*Sort1 (Nt-3), Fgf2, Cntf,* and *Bdnf*], apoptosis (*Casp3*), vascularization (*Vegfa*), axonal sprouting (*Gap43*), astrogliosis (*Gfap*), and oligodendrocytes (*Olig2*) was determined 10 and 28 days and 2 months after SCI (Fig 33B). Transplantation with SPC-01-GFP cells and iPSC-NPs did not result in any significant changes in gene expression at any of the time points. The greatest upregulation of gene expression was found in *Fgf2*. However, this change was not significant. Ten days after spinal cord injury (3 days after grafting), animals

implanted with MSCs displayed upregulation of all genes of interest in a strong, but insignificant trend (Fig. 33b1). This pattern of gene expression in the MSC treated group continued until the end of experiment at 28 days after SCI, with the exception of downregulation of *Casp3* (Fig. 33b2). Two months after SCI, the MSC group showed significant downregulation of *Bdnf*, *Vegf* and *Ngf* in when compared with saline treated and both NP treated groups (Fig. 33b3).

Expression of genes related to the macrophage phenotypes (*Irf5*, *Cd86*, *Mrc1* and *Cd163*) was determined 10 and 28 days after cell transplantation (Fig. 33A). Ten days after SCI, rats transplanted with MSCs displayed upregulation of *Irf5* and *Mrc1* when compared to the control animals. Gene expression remained unaltered in samples taken from the SPC-01-GFP treated rats. In contrast, iPSC-NP treated rats displayed downregulation of *Cd86* and *Cd163* (Fig 33a1). Three weeks after grafting, all genes related to both macrophage phenotypes were upregulated, however without statistical significance (Fig. 33a2).

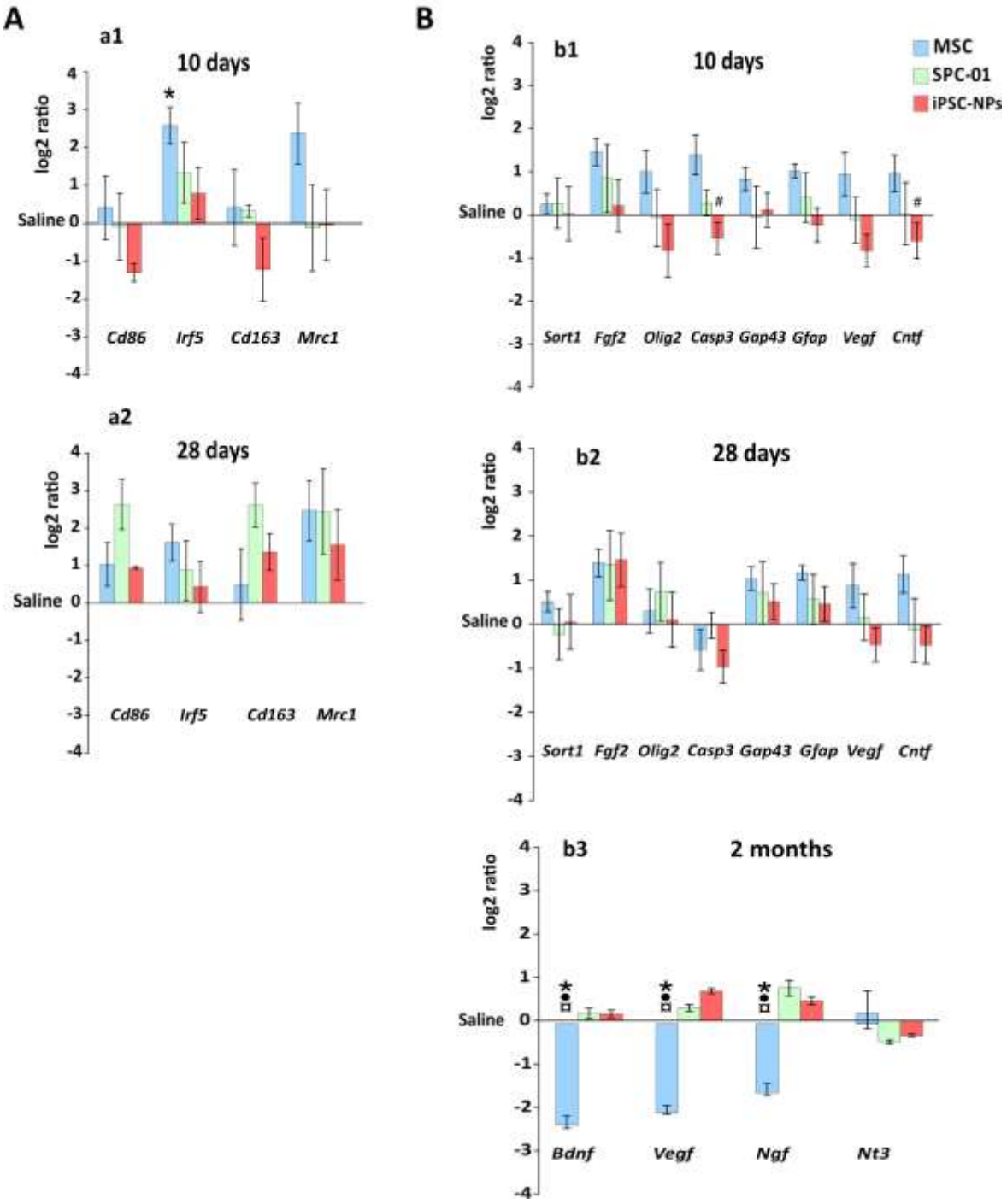

Fig. 33 Effect of stem cell implantation on gene expression of factors related to macrophage presence and phenotype and recovery after SCI. Changes of relative expression of macrophage phenotype related genes (M1-*Cd86, Irf5*; M2-*Cd163, Mrc1*) **(A)** in response to grafted MSCs, SPC-01, and iPSC-NP cells 10 **(a1)** and 28 **(a2)** days after SCI, demonstrating that the most significant changes were evident following MSC treatment, with upregulated *Irf5* 10 days after SCI. Expression levels measured in saline-treated rats were set to 0. Relative gene expression of factors related to recovery process **(B)** in response to grafted MSCs, SPC-01, or iPSC-NP cells 10 days **(b1)**, 28 days **(b2)**, and 2 months **(b3)** after SCI. The expression levels in saline-treated rats were set to 0. Data is presented as mean and SEM with $^{*}p < 0.05$ versus saline; $\#p < 0.05$ versus MSCs; $\bullet p < 0.05$ versus SPC-01; $\square p < 0.05$ versus iPSC-NPs.

4.3.5. Morphometry

Using Cresyl violet-Luxol fast blue staining, the area of spared white and gray matter was measured 2 months after SCI. Values were related to areas measured in saline treated control animals, set as 100%. Noticeable tissue sparing was detected as a result of transplantation of all types of stem cells. However, only the implantation of iPSC-NPs caused significant tissue preservation of white and gray matter when compared to saline controls (Fig. 34A, B).

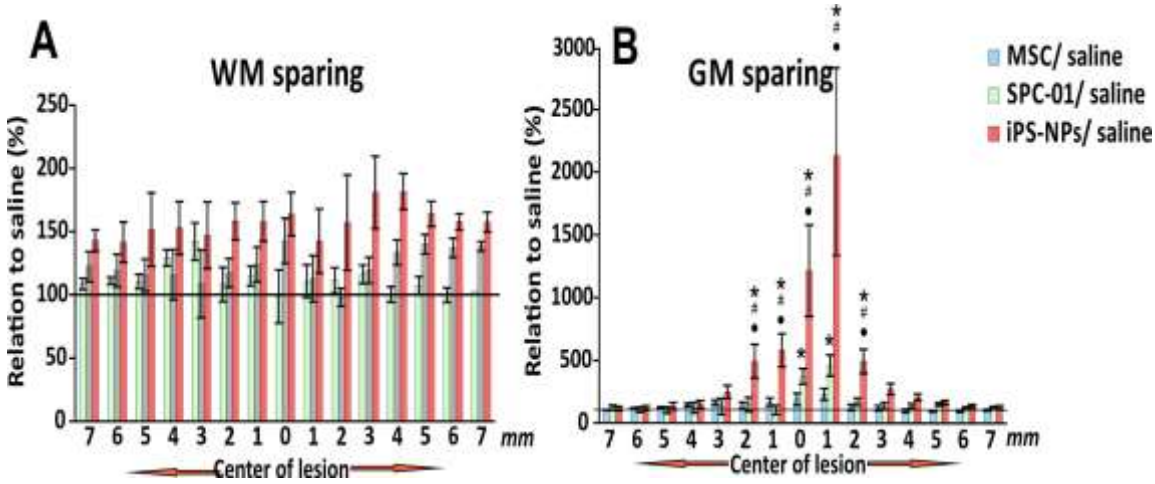

Fig. 34 Areas of spared white **(A)** and gray **(B)** matter were determined 2 months after SCI in rats treated with saline or stem cells (MSC, SPC-01, and iPSC-NP). All cell types had positive impact on tissue sparing in the WM/GM of the injured spinal cord. However, only the iPSC-NP group showed statistically significant preservation of spinal tissue. Data is presented as mean and SEM with $^{*}p < 0.05$ versus saline; $\#p < 0.05$ versus MSCs; $\bullet p < 0.05$ versus SPC-01

4.3.6. Glial scar and astrocyte reaction

Glial reaction to injury and treatment with stem cells was assessed by measuring the area of spinal tissue positive to GFAP. For this, immunofluorescent staining (anti GFAP-CY3) was used 2 months after SCI. Values are presented as a ratio (%) of the $GFAP^{+}$ area relative to the total tissue area. All stem cell treated groups displayed significantly smaller glial activation and $GFAP^{+}$ area volume when compared to saline controls. Additionally, transplantation of SPC-01-GFP cells or iPSC-NPs resulted in a significantly smaller $GFAP^{+}$ volume when compared with tissue from rats transplanted with MSCs (Fig. 35A). Moreover, rats injected with saline, MSCs, or SPC-01-GFP cells had a significantly higher number of protoplasmic astrocytes per slice in comparison with the iPSC-NPs group (Fig. 35B).

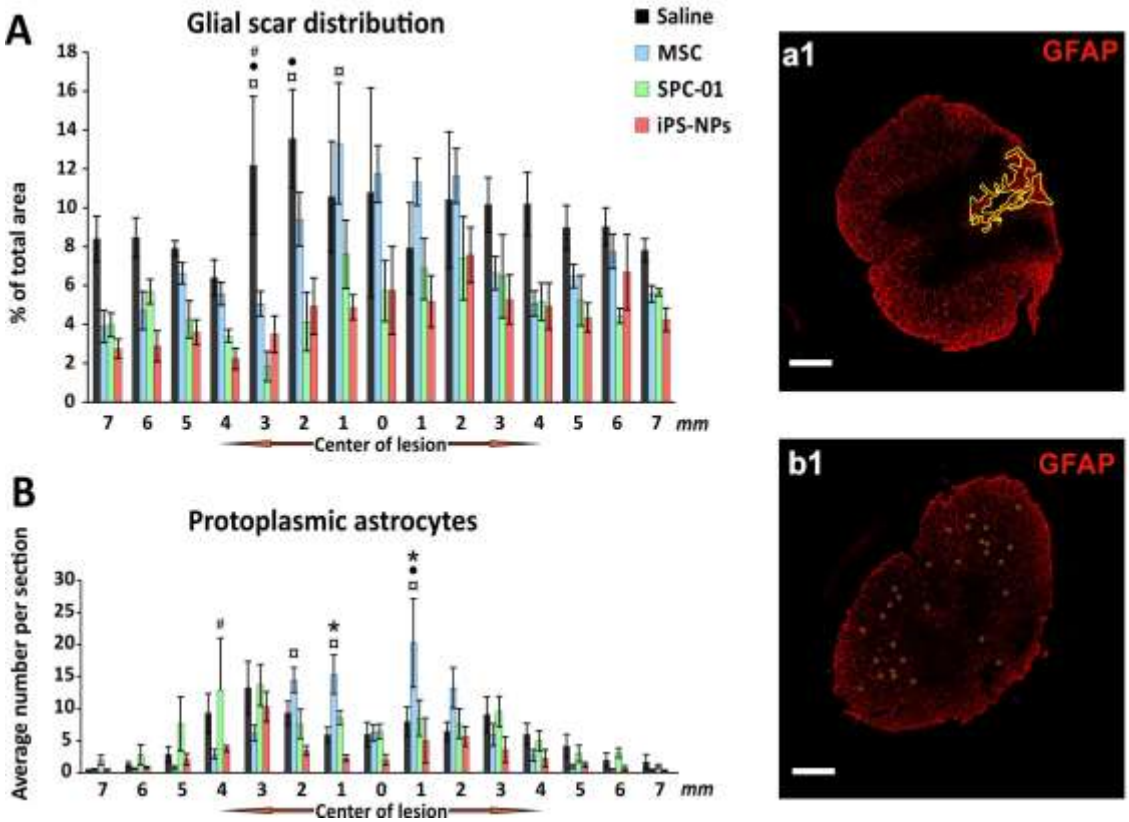

Fig. 35 Effect of implanted MSCs, SPC-01 and iPSC-NP cells on glial scar formation. Graphs depict the area of astrogliosis surrounding the main cavity as a percentage of the total tissue area **(A, a1)** and the average number of protoplasmic astrocytes per section **(B, b1)** around the central lesion cavity 9 weeks after SCI. Illustrative images show GFAP-CY3 staining of the glial scar **(a1)** and protoplasmic astrocytes **(b1)**. Scale bars: 500 µm. Both neural precursors reduced glial scaring and the number of protoplasmic astrocytes, whereas MSCs modulated the distribution of glial with only mild reduction. Data are presented as mean and SEM with $^{*}p < 0.05$ versus saline; #$p < 0.05$ versus MSCs; •$p < 0.05$ versus SPC-01; ¤$p < 0.05$ versus iPSC-NPs.

4.3.7. Axonal sprouting

Newly forming axons were detected by counting GAP43^{+} fibers in cross sections of spinal cords 2 months after injury. Values presented are related to the value found in saline treated animals, which was set as 100% (Fig. 36A). Transplantation of either type of neural precursors resulted in higher numbers of GAP43^{+} fibers, when compared to both saline treated and MSC treated rats (Fig. 36a1, a2).

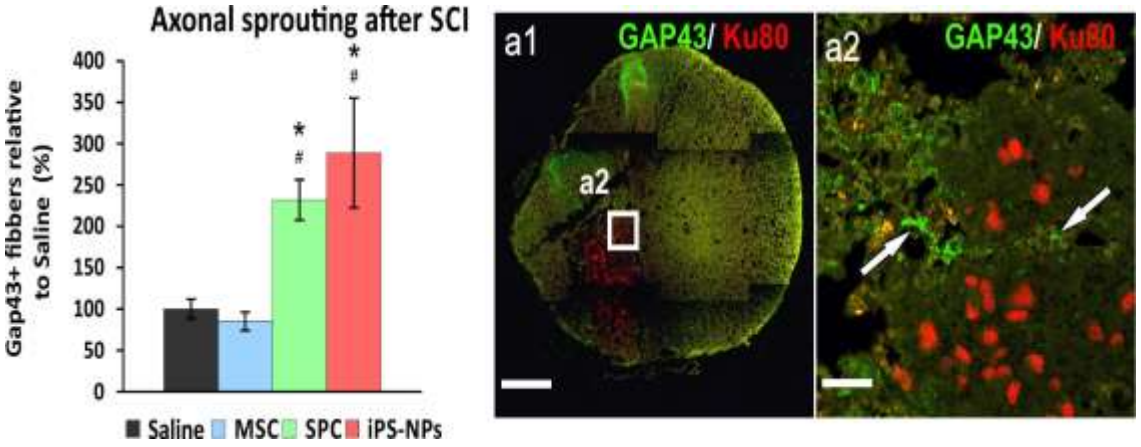

Fig. 36 Effect of stem cells on axonal sprouting **(A)**. Numbers of GAP43$^+$ fibers (15 sections per rat) are presented as percentage relative to the number of positive fibers after saline treatment (set to 100%). Representative images of a GAP43$^+$ labeled section **(a1, a2)** with grafted cells positive for nuclear marker Ku80, visualizing cells of human origin. Arrows mark the GAP43$^+$ fibers. Scale bars: 500 μm **(a1)**, 20 μm **(a2)**. Transplantation of both neural precursors resulted in increased axonal sprouting when compared to saline or MSCs treated animals. $^*p < 0.05$ versus saline; $\#p < 0.05$ versus MSCs; $\bullet p < 0.05$ versus SPC-01; $¤p < 0.05$ versus iPSC-NPs.

4.3.8. Functional outcomes

The BBB open field test was performed 1 week prior to spinal cord injury and then once every week for 9 weeks to evaluate hind limb locomotor recovery. Transplantation of either stem cell type was conducted 1 week after injury induction. All stem cell treated rats showed significantly better performance when compared with saline treated controls. Additionally, neural precursors derived from iPS cells and MSCs displayed superior potential to promote functional outcome than SPC-01-GPF cells (Fig. 37A).

Changes in the sensory function of the hind limb after SCI were measured using Plantar test. All stem cell-treated animals were similar to the saline-treated group, which implies that no additional hyperalgesia appeared as a result of stem cell transplantation (Fig. 37E).

Recovery of advanced locomotor function was evaluated using the flat beam test. In this test, performance of rats grafted with iPSC-NPs was superior to rats transplanted with other cell types, showing the greatest degree of improvement when compared to the saline treated animals. Animals injected with either SPC-01-GFP cells or mesenchymal stem cells did not significantly improve higher motor functions like coordination and weight support when compared to saline treated rats (Fig. 37B).

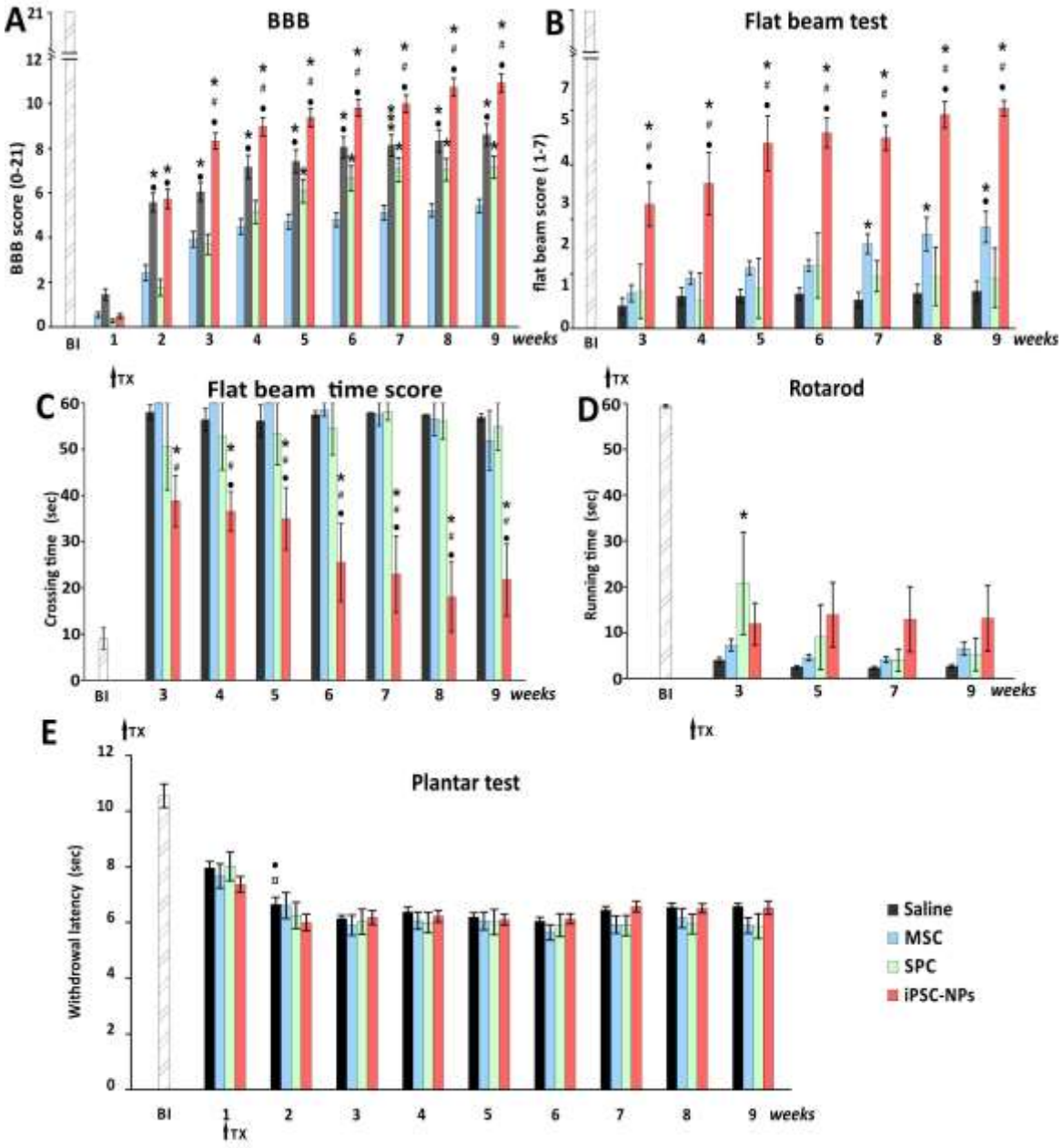

Fig. 37 Locomotor and sensory recovery resulting from stem cell transplantation after SCI was assessed using the BBB **(A)**, flat beam **(B)**, flat beam time score **(C)**, and rotarod tests **(D)**. All treated animals displayed significantly improved performances in the open-field BBB test in comparison with saline controls **(A)**. Additionally, the iPSC-NP and MSC groups demonstrated significantly higher performances compared to SPC-01-GFP group. In tests requiring more advanced locomotor skills, such as the flat beam test **(B)**, all treated groups performed significantly better when compared with saline control, with iPSC-NPS having the best results. In the rotarod test **(D)**, for which body weight supported stepping is essential, a stable but insignificant trend of iPSC-NPs in improved performance was observed. Thermal nociception was assessed using the plantar test **(E)**. No additional hyperalgesia after grafting of stem cells was observed. Data is presented as mean and SEM with $^{*}p < 0.05$ versus saline; $\#p < 0.05$ versus MSCs; $\bullet p < 0.05$ versus SPC-01; $¤p < 0.05$ versus iPSC-NPs. BI-before injury, TX-transplantation.

Time score in the flat beam test reflects the time it takes to cross the beam within a 60-s test period and requires higher level of coordination and muscle strength. The best performance was detected in rats grafted with iPSC-NPs showing the greatest improvement when compared to the saline treated animals. Performance of iPS-NPs treated group was superior to treatment groups treated with other stem cell types, showing progressive improvement over the course of experiment (Fig. 37C).

Rotarod test was used to determine long-term weight support, endurance and coordinated stepping. Due to high difficulty of the test, no significant differences in stem cell treated rats were observed when compared with rats treated with saline (Fig. 37D).

5. Discussion

Spinal cord injury is known to be a complex process accompanied by significant inflammatory response. By using compounds with proven anti-inflammatory and also anti-oxidant properties, we hypothesized that these compounds could modulate inflammation after SCI and thus help improve regeneration and functional outcome.

In this study, two anti-inflammatory compounds, EGCG or curcumin were administered *in situ* immediately after induction of spinal cord injury and then every day until the end of experiment. Several approaches were taken to evaluate changes in the immune response. The strongest evidence of these compounds altering immune response is inhibition of nuclear translocation of p65 protein into nuclei of cells in the spinal cord. Both EGCG and curcumin strongly reduced the activation of NFκB pathway containing p65 in the NFκB dimer, which is a typical constituent of canonical pathway. The observation that curcumin inhibits NFκB pathway was later confirmed by several groups (Gokce et al., 2016; Yuan et al., 2017; Zhang et al., 2017). Analysis of NFκB1 (p105) expression revealed significant downregulation at 10 day interval after curcumin treatment, this reduction of p105 (precursor of p50) later stabilized and was not different from controls like in the case of EGCG treatment. Injury to the central nervous system triggers NFκB pathway in neurons and glial cells, which results in several processes depending on the type of stimulus and also on the composition of NFκB dimer. NFκB dimers are present in inactive state in the cytoplasm inhibited by IκB molecules, which are phosphorylated and dissociated upon activation, enabling NFκB dimers to translocate into the nucleus to trigger gene transcription. Once its task in the nucleus is complete, it is transported out of the nucleus and can be recycled and used in new signaling. Thus our observation of downregulation or no change in expression of NFκB1 (p105), which is later processed into a common NFκB protein p50, is not indicative of reduction in activity of NFκB pathway containing this protein (including the much studied p50/p65 combination), but it rather suggests no increase of this pathway's activity. This correlates with our finding that both EGCG and curcumin reduce p65 nuclear translocation resulting in inhibition of canonical p50/p65 NFκB pathway. However, other NFκB dimers, containing p50, c-Rel, or other NFκB proteins are very likely still active, continuing to impact both immune reaction and neuroprotective processes. There is evidence, that dimers containing c-Rel trigger transcription of genes with rather neuroprotective roles. This is supported by the fact,

that c-Rel-/- mice develop Parkinsonism proving the crucial role of c-Rel in prevention of neurodegeneration (Baiguera et al., 2012). In trauma, NFκB transcription results in production of anti-apoptotic proteins but also pro-apoptotic FASL or cytokine TNFα, which can further activate the NFκB through TNFR1 or TNFR2 receptors. Together with IL-1β, this cytokine is the first to be detected after CNS trauma (Pan et al., 2002), has pro-apoptotic effect and also is involved in blood-spinal cord barrier disruption. TNFα has been shown to be cytotoxic to oligodendrocytes and thus its presence contributes to demyelination (Garcia et al., 2016). In our study, TNFα was reduced in animals treated with curcumin 1 day and 2 weeks after SCI, which was confirmed by other groups (Gokce et al., 2016; Zhang et al., 2017). Interleukins IL-1β, IL-6 or IL-12 are crucial in activation of phagocytosing cells and induce expression of adhesion molecules facilitating infiltration of immune cells from the periphery. While all these cytokines are implicated in prolonged, exaggerated immune response, which causes additional damage to the spinal cord and loss of functional connections, they are also essential in clearing out debris leading to wound healing and regeneration. Dimers containing p65 are associated with production of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) in microglia and infiltrated immune cells as well as in astrocytes. We found, that curcumin treatment decreased levels of IL-2 at all studied times except for 7 and 10 days after SCI. In contrast, we observed that levels of IL-6 and IL-12p70 increased over time with significantly higher levels at 14 and 28 days after injury when compared with control groups, which could be due to transcription by other transcription factors such as JAK2-STAT3 (Qian et al., 2014; Chen et al., 2017; Yang and Tang, 2017), STAT4 (Maier et al., 2002), AP-1 (Garcia et al., 2016) indicating that curcumin modulation may be targeted specifically at p50/p65. Recently, Yang and Tang discovered, that presence of IL-6 inhibits expression of inhibiting myelin proteins Nogo-A and its receptor NgR and strongly increases expression of GAP43 involved in new axonal growth. This effect was dose-dependent and the role of IL-6 in structural and functional recovery was confirmed by robust improvement in BBB test, highlighting the dual role of IL-6 (Yang and Tang, 2017). Interestingly, Yaguchi et al. reported functional recovery in mice after implanting a gelfoam sponge containing IL-12 *in situ* (Yaguchi et al., 2008). This suggests that increased levels of IL-12 may be important in achieving motor improvements and has indeed been reported to decrease in the first 24 hours after SCI in rodents (Stammers et al., 2012). In our analysis, we also observed low levels of IL-12p70 after SCI, but this was not significantly lower when

compared to healthy animals. Treatment with EGCG also resulted in marked immunomodulation. Production of TNFα and IL-12 was initially increased but gradually decreased. Additionally, chemokines MIP1α, RANTES and IL-6 were strongly reduced throughout the first week, but levels of these chemokines later increased. Contrary to our findings, reduction of TNFα 24 hours after SCI was detected by Khalatbary et al. (Khalatbary and Ahmadvand, 2011). This disagreement could be due to different methodology (IHC vs Luminex). Inhibition of canonical p50/p65 pathway resulted in significant modulation of cytokine production profile after each treatment. However, in case of EGCG, this effect was strongest in the first 24 hours and gradually disappeared, with the exception of upregulation of neuroprotective IL-4 (Francos-Quijorna et al., 2016; Lima et al., 2017) at 14 days after SCI. In contrast, curcumin decreased inflammatory cytokine and chemokine production throughout the entire experiment, correlating with decreased NFκB activity, especially at early and later time points, in which we demonstrated the highest levels of p65 nuclear translocation. Several other groups demonstrated immunomodulatory and anti-apoptotic properties of curcumin (Gokce et al., 2016; Yuan et al., 2017; Zhang et al., 2017) or EGCG (Khalatbary and Ahmadvand, 2011; Tian et al., 2013). We found, that presence of IL-4, cytokine exerting anti-inflammatory effects after trauma to CNS through prevention of microglia over-activation (Yang et al., 2002; Park et al., 2005) was either unchanged or elevated after both EGCG and curcumin treatments adding to the overall inhibitory effect of tested compounds. Changes in production of studied cytokines had significant impact on the surrounding tissue, which may have resulted in significantly enhanced axonal sprouting after both treatments and in case of curcumin, also in smaller cavity size, which can be explained by increased levels of IL-6 in animals treated with curcumin. Higher levels of $GAP43^{+}$ fibers after EGCG treatment were also observed by Renno et al. (Renno et al., 2014) suggesting that EGCG supports axonal sprouting by another mechanism. Moreover, we discovered that EGCG treatment significantly altered the phenotype of resident or infiltrated macrophages. At 10 days into the treatment with EGCG and curcumin, we detected prevalence of phenotype associated more with classical M1 macrophages (*Cd86, Irf5*), which are involved in more acute phase of immune reaction, needed to clear out debris from damaged tissue, preventing exacerbation of immune response. When their presence is prolonged, these cytokine factories may be detrimental to spared tissue. This was prevented after treatment with EGCG, which caused reduction of expression of these markers as well as increase in

Cd163 and *Mrc1* markers associated with M2 macrophages known to facilitate healing and tame the immune reaction at later time point (Ji et al., 2015; Veremeyko et al., 2018). We did not detect such effect on macrophages after curcumin treatment. However, we found low expression levels of *Cd86* and *Irf5* genes. GFAP expression was also altered in studies, where treatment with anti-inflammatory compounds resulted in smaller $GFAP^+$ area in central parts of spinal cord lesion. Moreover, treatment with curcumin downregulated *Gfap* transcription. This effect was found by other groups who used curcumin (Zu et al., 2014; Yuan et al., 2017) or EGCG (Renno et al., 2014). Upregulation of GFAP expression marks an increase in astrocyte reactivity, which is, among other things, characterized by production of cytokines typical in NFκB transcription, such as IL-6, IL-1β, TNFα and COX-1 or iNOS (Bellaver et al., 2017; Tjalkens et al., 2017). Decrease in astrocytic reactivity can also be a result of NFκB pathway inhibition, even though NFκB is not directly involved in *Gfap* transcription. Taken together, all these molecular changes due to anti-inflammatory treatment strategy may have ultimately resulted in enhanced locomotor function as demonstrated in experiments using EGCG or curcumin. Interestingly, despite both compounds having profound effects on gene expression and protein production, the underlying mechanisms are different as the ultimate effect in functional outcome of curcumin was noticeable in BBB open field test while EGCG recovered more complex motor function detected with flat beam test.

In the second part of our study, we focused on treatment of spinal cord injury with bone marrow mesenchymal stem cells, spinal neural precursors SPC-01 and neural precursors derived from induced pluripotent stem cells. We hypothesized that all types of cells used in this study would enhance recovery after SCI with MSCs acting mainly through paracrine effect on host tissue and immunomodulation. We expected neural precursors (NPs) to have better survival and remain in contact with host spinal cord to restore function by new axonal growth, cell replacement as well as modulation of immune responses. We chose to transplant either cell type 7 days after induction of spinal cord injury - postponed implantation of cells is a strategy adopted by many groups (Nori et al., 2011; Plemel et al., 2011; Tsuji et al., 2011; Kobayashi et al., 2012; Emgard et al., 2014; Lu et al., 2014). It has been shown by Cheng et al., that in case of neural stem cells transplantation, the best results were observed when cells were implanted sub-acutely 7 days after SCI (Cheng et al., 2017). Better outcomes after acute or sub-acute

cells administration was previously confirmed in meta-analysis conducted by the Hosseini group (Yousefifard et al., 2016).

In our studies, only a small number of transplanted MSCs survived beyond two weeks after implantation via intrathecal injection. Findings of progressive decline in presence of transplanted MSCs after SCI were reported by several groups (Boido et al., 2014; Torres-Espin et al., 2014; Torres-Espin et al., 2015; Yousefifard et al., 2016). Yousefifard et al. observed survival of 0.36% out of 1 million transplanted cells 8 weeks after transplantation (Yousefifard et al., 2016). Torres-Espin et al. also observed progressive decline of survival 2 weeks after transplantation, which did not depend on the number of cells transplanted - 0.5 vs 1 million cells (Torres-Espin et al., 2015). Similar survival rate and kinetics were observed when 0.5 million MSCs were implanted regardless whether it was done 30 mins or 7 days after SCI (Torres-Espin et al., 2014). Survival rates were improved when immunosuppressant tacrolimus was used and roughly 50% more cells survived when compared to controls (Torres-Espin et al., 2015). These results indicate, that transplanted MSCs are indeed immunogenic and trigger rejection reaction, which was confirmed by elevated genes involved in such response (Torres-Espin et al., 2013) and may be the principal reason for low survival rates of MSCs transplanted into injured spinal cords. However, we also used immunosuppression with all our cells and still observed low survival of MSCs. In the above mentioned studies, scientists opted for *in situ* application of MSCs, whereas we used a less invasive approach, which we found to be similarly effective in SCI treatment as spinal injection (Amemori et al., 2010), but which could have had effect on low survival. Contrary to MSCs, we found much better survival of both types of neural precursors, which was confirmed 2 months after grafting. Good survival of implanted NPs has been reported by other groups (Yan et al., 2007; Emgard et al., 2014; Wilcox et al., 2014; Brock et al., 2017). Additionally, Lu et al. conducted a long-term experiment with rats transplanted with human neural stem cells surviving for 1.5 years. They found that while maintaining its size, the graft continued maturation into all three neuronal lines and extended numerous axons half of which survived until the end of experiment. Notably, mature oligodendrocytes were not present until 1 year after transplantation, which is when functional recovery began (Lu et al., 2017). Results of this study highlight the need for more long-term studies to be conducted in order to observe complete effects of transplanted cells.

In all cases of cell therapy conducted by our group, we found significant functional improvements assessed by BBB open field test. By far, the best results were observed in animals transplanted with iPSC derived neural precursors, which was also apparent in Flat beam test score and reduced time it took to traverse the beam. Functional recovery in treatment of SCI with iPSCs-derived neural precursor cells has been found in other studies in mice (Nori et al., 2011; Tsuji et al., 2011) and also in non-human primates (Kobayashi et al., 2012). Improvements in motor function have been documented in studies using spinal neural progenitor cells (Brock et al., 2017) as well as bone marrow-derived mesenchymal stem cells (Fonseca et al., 2017; Salem et al., 2017; Xiong et al., 2017), which is in line with our findings. Exact mechanisms behind enhanced motor function and recovery are yet to be fully understood.

Transplantation of either cell type resulted in changes in the immune response of host tissue, which was determined by assessment of levels of p65 nuclear translocation, cytokine production and macrophage phenotype distinction. After spinal cord injury, the activity of NFκB pathway is significantly elevated when compared with healthy animals (data not shown) and triggers transcription of pro-inflammatory, pro-apoptotic proteins as well as healing promoting and anti-apoptotic ones. We observed that p65 nuclear translocation has two peaks in the time frame and end points of our experiment. Most significant elevations were detected 3 days and 28 days after induction of spinal cord injury, which validates the approach of postponed transplantation. Analysis of NFκB activation in cell treated animals revealed, that both spinal neural precursors SPC-01 and mesenchymal stem cells significantly reduced levels of p65 nuclear translocation 28 days after SCI with SPC-01 cells being the most potent NFκB inhibitors. Interestingly, iPS-derived neural precursors displayed little effect on NFκB activation. In groups transplanted with iPS-NPs, we found no difference when compared with saline controls 3, 7 and 21 days after transplanting. However, the expression profile of macrophage surface markers at 21 days after transplantation suggested that present macrophages were polarized more towards healing promoting macrophages in SPC-01 group. The expression of *Irf5* was low while *CD163* and *Mrc1* were both increased, albeit insignificantly. Interestingly, MSCs significantly increased expression of *Irf5* suggesting stronger presence of classical pro-inflammatory macrophages, however, this activation/recruitment was later reduced by roughly 40%. This reduction corresponds with significant inhibition of NFκB (p65) nuclear translocation, which taken together with decreased Casp3 production may be one of the underlying mechanisms of

therapeutic effect of MSCs confirmed by improved scores of BBB and flat beam tests. Clear immunomodulatory effect of MSCs was demonstrated in our experiments. Analysis of cytokine production showed marked reduction in levels of MIP1α, IL-2, TNFα as well as insignificant reduction in levels of IL-6, IL-12p70 cytokines. Rosado et al. showed similar effects of cryopreserved MSCs on host immune reaction. In their experiments, group transplanted with MSCs was found to have lowered levels of infiltrated macrophages, TNFα and GFAP, which is in line with our results (Rosado et al., 2017). Decrease of TNFα was also reported by Salem et al. (Salem et al., 2017). This group also demonstrated enhanced effects of MSCs when combined with anti-oxidant vitamin C. In our study, inhibiting production of cytokines was most pronounced in the first week after transplantation, perhaps due to little survival of MSCs beyond this point. Despite of low survival, their effect was far reaching, which was documented not only with reduction in glial scar and locomotor improvements at later stages, but also by inhibition of NFκB activity 21 days after their transplantation. However, we did not detect improved tissue sparing, axonal sprouting or changes in regeneration-associated genes, which suggests that therapeutic effect was in early immunomodulatory intervention, confirmed by Hosseini et al. who conducted combination therapy of spinal cord injury implanting MSCs immediately after SCI and neural stem cells 3 days later. This early modulation of host inflammatory reaction resulted in dramatic functional improvements of rats transplanted with both types of stem cells (Hosseini et al., 2018).

The best functional outcome was achieved after treatment with iPS-NPs. Improvements of locomotor function has been documented by other groups in mice and non-human primates (Kobayashi et al., 2012; Tang et al., 2013; Oh et al., 2015; Salewski et al., 2015). In line with findings of other groups, iPS-NPs displayed good survival and integration (Tang et al., 2013; Salewski et al., 2015) throughout experiment and caused several molecular changes in the host tissue that contributed to the therapeutic effect. We observed significant gray matter sparing in the lesion area of rats transplanted with iPS-NPs, which together with marked axonal regrowth may have resulted in enhanced signal transmission and thus preserved/gained function 2 months after SCI. Similar effect of iPS-NPs on tissue preservation along with functional recovery was found by Oh et al. in mice after transplantation of $5x10^5$ iPS-NPs (Oh et al., 2015). Enhanced axonal sparing/regrowth determined by total area positive for NF200 was detected by Kobayashi et al. in a model of SCI in common marmoset (Kobayashi et al., 2012).

Effect on axonal growth was found in our experiments, where we detected marked increase in newly sprouting GAP43^{+} axons and which was documented by other groups (Hong et al., 2014; Fu et al., 2017). Additionally, group of Fehlings demonstrated, that the primary mechanism of functional recovery in mice after treatment with iPS-NPs is through remyelination (Salewski et al., 2015). However, we did not detect statistically significant white matter sparing in our study, which could be attributed to different primary source and different protocol of iPS cells generation. In our experiments, iPS-NPs reduced levels of pro-inflammatory IL-1β, IL-2, TNFα and chemokines MIP1α and RANTES. This anti-inflammatory effect was accompanied by strong elevation in production of IL-6 and IL-12p70 at all time points, which supports findings of Yang and Tang that IL-6 promotes axonal sprouting characterized by presence of GAP43 (Yang and Tang, 2017) and by Yaguchi et al. who reported functional improvement after IL-12 administration (Yaguchi et al., 2008). Together with significant increase of IL-6 and IL-12p70 we found significant increase of NFκB, but only at 21 days after transplantation indicating that production of IL-6 and IL-12p70 is regulated by other transcription factors in spinal cord injury. Hong et al. transplanted induced neural stem cells 9 days after SCI and found strong immunomodulatory effect with reductions of IL-1β, TNFα, but also IL-6, which is where our results differ (Hong et al., 2014). Animals treated with iPS-NPs displayed increased production of IL-4, which has been proposed to have neuroprotective properties (Yang et al., 2002; Park et al., 2005; Lima et al., 2017).

Similarly to iPS-NP, transplantation of spinal neural precursors resulted in down- or upregulation of the same cytokines and chemokines. Reduction of IL-2, TNF-α, IL-1β and MIP1α and RANTES contributed to lowered immune reaction. Production of neuroprotective cytokine IL-4 was increased during the entire experiment, which together with significantly increased axonal sprouting contributed to functional improvements. Locomotor recovery was previously also demonstrated by Emgard et al. who transplanted neural progenitors as neurospheres (Emgard et al., 2014) or by Brock et al. who transplanted embryonic spinal cord-derived neural progenitor cells, which robustly extended newly formed axons and formed synapses with host neurons (Brock et al., 2017). Although insignificant, we observed a trend in macrophage phenotype shifting towards healing promoting macrophages with higher levels of *Mrc1* and low levels of *Irf5* markers 21 days after transplantation. Cheng et al. observed immunomodulatory effect of neural stem cells in mice where decreased levels of TNFα, IL-1β, IL-6 and IL-12 were accompanied with significant reduction of neutrophil and

macrophage infiltration. The group observed migration of the graft into lesion center and improvement of the BMS score. Interestingly, they found their neural stem cells to inhibit iNOS expression in macrophages *in vitro* as well (Cheng et al., 2016). Hosseini et al. also reported improved BBB along with decreased IL-1β but not IL-6 (Hosseini et al., 2018). In our study axonal growth promoting IL-6 along with IL-12p70 were strongly upregulated throughout the entire experiment, while activity of NFκB was at low levels and significantly reduced 21 days after SPC-01 transplantation. Increased levels of IL-6 and IL-12p70 were also observed in rats treated with iPS-NPs and in rats treated with curcumin, which strongly inhibited p65 nuclear translocation in all studied time points. This further supports the theory that regulation of IL-6 and IL-12p70 production is primarily or redundantly conducted by other transcription factor(s), such as JAK2-STAT3 (Qian et al., 2014; Chen et al., 2017; Yang and Tang, 2017), STAT4 (Maier et al., 2002) or AP-1 (Garcia et al., 2016).

6. Conclusion

We attempted to answer several questions in our studies regarding therapeutic effects of anti-inflammatory compounds EGCG or curcumin and effects of stem cell transplantation in the balloon compression model of spinal cord injury in rats. We found significant functional improvements in all treated groups out of which the best recovery was seen in iPS-NPs group. Animals treated with curcumin displayed robust improvement in the first week, which continued until the end of experiment. EGCG as well as spinal progenitors SPC-01 on the other hand improved motor function in later stages. To understand mechanisms behind these improvements, we tested an array of molecular targets to see whether they are affected by treatments and contribute to enhanced functional outcomes.

In these projects, we focused on immune reaction after spinal cord injury and how it is modulated after our treatments. We were specifically interested in the activity of transcription factor NFκB, which has a significant role in inflammation throughout the body and has been shown to be activated after trauma to the central nervous system. We found, that NFκB activity peaked at 3 and 28 days after SCI and is significantly lower at 1, 7, 10 and 14 days after SCI. This confirms that the host environment may be more permissible to begin cell therapy between 7 and 14 days after injury. We confirmed our hypothesis that phytochemicals EGCG and curcumin inhibit nuclear translocation of p65 in all studied time points with curcumin essentially preventing p65 to enter the

nucleus. Grafting of either cell type did not induce enhanced activation of NFκB pathway when compared to controls, however, we found significant inhibition of its activity in animals transplanted with SPC-01 and MSC cells, both at 28 days after SCI. Modification of p65 nuclear translocation in host cells likely contributed to recovery of transplanted animals or animals treated with phytochemicals. Apart from analysis of p65, we measured levels of a battery of cytokines to further determine the immunomodulatory effect of treatments. Most cytokines were altered after either treatment. To highlight the most pronounced effects, we observed a decrease in levels of TNFα, IL-2 and chemokines MIP1α and RANTES as well as an increase in IL-6 and IL-12p70 after treatment with curcumin. After using EGCG, we detected lower chemokine levels and also increased levels of neuroprotective IL-4. Additionally, IL-1β increased 14 days after SCI and TNFα and IL-12p70 were increased on the first day, which subsided over time. Another strong alteration that occurred after EGCG treatment was a shift in macrophage phenotype from classical to healing promoting, which was characterized by increased expression of especially mannose receptor 1 (*Mrc1)*. Stem cell treatments resulted in changes of cytokine production, notably a decrease of TNFα, IL-1β and chemokines. Interestingly, IL-6 and IL-12p70 were significantly increased in animals treated with either neural precursors or curcumin.

One of the aims of our study was to determine the regenerative potential of our treatments outside of altering the immune reaction. Together with morphometry, we determined transcription levels of genes associated with neural growth and differentiation, angiogenesis, apoptosis and expression of proteins involved in axonal sprouting or glial activation. Both curcumin and EGCG increased axonal growth seen in high expression of GAP43, a protein associated with neural growth cones. We also found this effect in animals treated with either NPs. This effect on axonal sprouting was caused by high levels of IL-6 except for animals treated with EGCG, where different mechanism had to be in play. Additionally, all treatments except for EGCG resulted in significantly lower astrogliosis making the host environment more permissible to new growth. Expressions of genes of interest varied from control animals. Significant changes were detected in group treated with MSC cells, where decrease in *Bdnf*, *Vegf* and *Ngf* was detected 2 months after grafting. Increased expression of VEGF, an indicator of enhanced angiogenesis was also found in animals treated with EGCG.

7. Summary

Taken together, our results confirm anti-inflammatory and/or immunomodulatory properties of curcumin and EGCG, as well as mesenchymal stem cells and both types of neural precursors. Only iPS-NPs failed to inhibit the canonical NFκB pathway. The fact that both SPC-01 cells and curcumin inhibited NFκB and iPS-NPs didn´t, but that in all these groups IL-6 and IL-12p70 were strongly elevated, prompts us to suggest that these cytokines are produced under different transcription factor(s) in spinal cord injury. Based on literature research, the likely candidate is JAK/STAT3. We observed decreased levels of some of the pro-inflammatory cytokines (TNFα, IL-1β, IL-2) in most treated animals as well as reduction in chemokine production. Based on our results confirmed by findings of other groups, we detected upregulation of IL-6 in animals treated with NPs or curcumin, which correlated with strong axonal sprouting. Despite low levels of IL-6, axonal sprouting was significantly enhanced in EGCG treated group. Neuroprotective IL-4 was highly produced after treatments with EGCG and both NPs, while production of protective IL-12p70 was significantly increased in all treated animals, but in case of EGCG its levels gradually decreased. Both NPs and curcumin treatment has limited effect on macrophage phenotype, unlike EGCG where we saw a shift towards healing promoting M2 macrophages and decrease in gene expression of classical macrophage marker *Irf5*. All treated animals except for MSCs and EGCG groups displayed lowered astrocyte activation partially alleviating inhibitory environment of host tissue. However, EGCG treatment resulted in upregulation of FGF and VEGF regeneration promoting growth factors. Elevated production of neurotrophic factors BDNF, NGF and angiogenesis promoting VEGF was observed in animals treated with SPC-01 neural precursors. In our studies, we demonstrated varying immunomodulatory and regenerative properties of tested therapies, which all resulted in marked functional recovery with iPS-NPs displaying the best motor improvements.

8. Souhrn

Naše výsledky potvrzují protizánětlivé a/nebo imunomodulační vlastnosti kurkuminu a EGCG, ale i mezenchymálních kmenových buněk a obou typů neurálních prekurzorů. Pouze neurální prekurzory derivované z iPS buněk nedokázaly zabránit aktivaci klasické signalizační dráhy transkripčního faktoru NFκB. Skutečnost, že kromě iPS-NPs byla NFκB dráha inhibována jak SPC-01 buňkami, tak kurkuminem při vysoké produkci cytokinů IL-6 a IL-12p70 u všech třech léčebných postupů, podporuje naši teorii, že tyto cytokiny jsou při poranění míchy primárně či redundantně transkribovány

pod jiným faktorem. Na základě studia literatury se jako pravděpodobná jeví JAK/STAT3 dráha. U většiny léčených zvířat jsme pozorovali jak snížené hladiny některých prozánětlivých cytokinů (TNFα, IL-1β, IL-2), tak snížení produkce chemokinů. Na základě našich výsledků potvrzených výsledky ostatních skupin jsme zjistili zvýšení regulace IL-6 u zvířat léčených neurálními prekurzory nebo kurkuminem, která navíc koreluje se silným růstem axonálních kolaterál. Navzdory nízkým hladinám IL-6 byl růst axonů významně zvýšen i u skupiny léčené EGCG. Neuroprotektivní IL-4 byl vysoce produkován po léčbě pomocí EGCG i obou neurálních prekurzorů, zatímco produkce proteinu IL-12p70 byla významně zvýšena u všech léčených zvířat, ale v případě EGCG se jeho hladina postupně snižovala. Oba typy neurálních prekurzorů a léčba kurkuminem mají omezený účinek na makrofágový fenotyp. Naproti tomu po aplikaci EGCG jsme zaznamenali silný posun k hojení podporujícím makrofágům M2 a po podání buněk MSC zase pokles exprese genu markeru klasických makrofágů *Irf5*. Všechna zvířata s výjimkou skupin MSC a EGCG vykazovala sníženou reaktivitu astrocytů, což mělo za následek zmírnění inhibičního prostředí poškozené míšní tkáně potkanů.

Léčba EGCG vedla ke zvýšené expresi růstových faktorů podporujících regeneraci FGF a VEGF. Zvýšená produkce neurotrofických faktorů BDNF, NGF a faktoru VEGF podporujícího angiogenezi byla pozorována u zvířat léčených neurálními prekurzory SPC-01. V našich studiích jsme prokázali různé imunomodulační a regenerační vlastnosti testovaných terapií, které vedly k výraznému funkčnímu zotavení. Nejlepší výsledek behaviorálního testování jsme zaznamenali po léčbě pomocí iPS-NP, kdy došlo k výraznému zlepšení motoriky.

9. Literature

. from https://rockland-inc.com/nfkb-signaling-pathway.aspx.

Ahmed, A., Patil, A. A. and Agrawal, D. K. (2017). "Immunobiology of spinal cord injuries and potential therapeutic approaches." Mol Cell Biochem.

Amemori, T., Jendelova, P., Ruzickova, K., Arboleda, D. and Sykova, E. (2010). "Co-transplantation of olfactory ensheathing glia and mesenchymal stromal cells does not have synergistic effects after spinal cord injury in the rat." Cytotherapy **12**(2): 212-225.

Amemori, T., Romanyuk, N., Jendelova, P., Herynek, V., Turnovcova, K., Prochazka, P., Kapcalova, M., Cocks, G., Price, J. and Sykova, E. (2013). "Human conditionally immortalized neural stem cells improve locomotor function after spinal cord injury in the rat." Stem Cell Res Ther **4**(3): 68.

Anderson, M. A., Burda, J. E., Ren, Y., Ao, Y., O'Shea, T. M., Kawaguchi, R., Coppola, G., Khakh, B. S., Deming, T. J. and Sofroniew, M. V. (2016). "Astrocyte scar formation aids central nervous system axon regeneration." Nature **532**(7598): 195-200.

Anderson, M. A., Burda, J. E., Ren, Y. L., Ao, Y., O'Shea, T. M., Kawaguchi, R., Coppola, G., Khakh, B. S., Deming, T. J. and Sofroniew, M. V. (2016). "Astrocyte scar formation aids central nervous system axon regeneration." Nature **532**(7598): 195-+.

Assinck, P., Duncan, G. J., Hilton, B. J., Plemel, J. R. and Tetzlaff, W. (2017). "Cell transplantation therapy for spinal cord injury." Nat Neurosci **20**(5): 637-647.

Badaut, J., Ashwal, S. and Obenaus, A. (2011). "Aquaporins in cerebrovascular disease: a target for treatment of brain edema?" Cerebrovasc Dis **31**(6): 521-531.

Baiguera, C., Alghisi, M., Pinna, A., Bellucci, A., De Luca, M. A., Frau, L., Morelli, M., Ingrassia, R., Benarese, M., Porrini, V., Pellitteri, M., Bertini, G., Fabene, P. F., Sigala, S., Spillantini, M. G., Liou, H. C., Spano, P. F. and Pizzi, M. (2012). "Late-onset Parkinsonism in NFkappaB/c-Rel-deficient mice." Brain **135**(Pt 9): 2750-2765.

Bajrektarevic, D. and Nistri, A. (2017). "Ceftriaxone-mediated upregulation of the glutamate transporter GLT-1 contrasts neurotoxicity evoked by kainate in rat organotypic spinal cord cultures." Neurotoxicology **60**: 34-41.

Basso, D. M., Beattie, M. S. and Bresnahan, J. C. (1995). "A sensitive and reliable locomotor rating scale for open field testing in rats." J Neurotrauma **12**(1): 1-21.

Bellaver, B., Dos Santos, J. P., Leffa, D. T., Bobermin, L. D., Roppa, P. H. A., da Silva Torres, I. L., Goncalves, C. A., Souza, D. O. and Quincozes-Santos, A. (2017). "Systemic Inflammation as a Driver of Brain Injury: the Astrocyte as an Emerging Player." Mol Neurobiol.

Bellaver, B., Dos Santos, J. P., Leffa, D. T., Bobermin, L. D., Roppa, P. H. A., da Silva Torres, I. L., Goncalves, C. A., Souza, D. O. and Quincozes-Santos, A. (2017). "Systemic Inflammation as a Driver of Brain Injury: the Astrocyte as an Emerging Player." Molecular Neurobiology: 1-11.

Biasibetti, R., Tramontina, A. C., Costa, A. P., Dutra, M. F., Quincozes-Santos, A., Nardin, P., Bernardi, C. L., Wartchow, K. M., Lunardi, P. S. and Goncalves, C. A. (2013). "Green tea (-)epigallocatechin-3-gallate reverses oxidative stress and reduces acetylcholinesterase activity in a streptozotocin-induced model of dementia." Behav Brain Res **236**(1): 186-193.

Birck, C., Koncina, E., Heurtaux, T., Glaab, E., Michelucci, A., Heuschling, P. and Grandbarbe, L. (2016). "Transcriptomic analyses of primary astrocytes under TNFalpha treatment." Genom Data **7**: 7-11.

Birkinshaw, R. W. and Czabotar, P. E. (2017). "The BCL-2 family of proteins and mitochondrial outer membrane permeabilisation." Semin Cell Dev Biol.

Boido, M., Garbossa, D., Fontanella, M., Ducati, A. and Vercelli, A. (2014). "Mesenchymal stem cell transplantation reduces glial cyst and improves functional outcome after spinal cord compression." World Neurosurg **81**(1): 183-190.

Boido, M., Garbossa, D. and Vercelli, A. (2011). "Early graft of neural precursors in spinal cord compression reduces glial cyst and improves function." J Neurosurg Spine **15**(1): 97-106.

Bonner, J. F., Connors, T. M., Silverman, W. F., Kowalski, D. P., Lemay, M. A. and Fischer, I. (2011). "Grafted neural progenitors integrate and restore synaptic connectivity across the injured spinal cord." J Neurosci **31**(12): 4675-4686.

Bouvier, G., Astri, S., Orsini, N., Kunze, G., Luzy, A. P. and Gross, D. (2017). "Different damage response of cis and trans isomers of commonly used UV filter after the exposure on adult human liver stem cells and human lymphoblastoid cells: A response." Sci Total Environ **625**: 262-263.

Brock, J. H., Graham, L., Staufenberg, E., Im, S. and Tuszynski, M. H. (2017). "Rodent Neural Progenitor Cells Support Functional Recovery After Cervical Spinal Cord Contusion." J Neurotrauma.

Busch, S. A., Hamilton, J. A., Horn, K. P., Cuascut, F. X., Cutrone, R., Lehman, N., Deans, R. J., Ting, A. E., Mays, R. W. and Silver, J. (2011). "Multipotent adult progenitor cells prevent macrophage-mediated axonal dieback and promote regrowth after spinal cord injury." J Neurosci **31**(3): 944-953.

Cantinieaux, D., Quertainmont, R., Blacher, S., Rossi, L., Wanet, T., Noel, A., Brook, G., Schoenen, J. and Franzen, R. (2013). "Conditioned medium from bone marrow-derived mesenchymal stem cells improves recovery after spinal cord injury in rats: an original strategy to avoid cell transplantation." PLoS One **8**(8): e69515.

Capetian, P., Azmitia, L., Pauly, M. G., Krajka, V., Stengel, F., Bernhardi, E. M., Klett, M., Meier, B., Seibler, P., Stanslowsky, N., Moser, A., Knopp, A., Gillessen-Kaesbach, G., Nikkhah, G., Wegner, F., Dobrossy, M. and Klein, C. (2016). "Plasmid-Based Generation of Induced Neural Stem Cells from Adult Human Fibroblasts." Frontiers in Cellular Neuroscience **10**: 245.

Cregg, J. M., DePaul, M. A., Filous, A. R., Lang, B. T., Tran, A. and Silver, J. (2014). "Functional regeneration beyond the glial scar." Experimental Neurology **253**: 197-207.

D'Orsi, B., Mateyka, J. and Prehn, J. H. (2017). "Control of mitochondrial physiology and cell death by the Bcl-2 family proteins Bax and Bok." Neurochem Int.

Dhandapani, K. M., Wade, F. M., Wakade, C., Mahesh, V. B. and Brann, D. W. (2005). "Neuroprotection by stem cell factor in rat cortical neurons involves AKT and NFkappaB." J Neurochem **95**(1): 9-19.

Diaz-Ruiz, A., Montes, S., Salgado-Ceballos, H., Maldonado, V., Rivera-Espinosa, L. and Rios, C. (2016). "Enzyme activities involved in the glutamate-glutamine cycle are altered to reduce glutamate after spinal cord injury in rats." Neuroreport **27**(18): 1317-1322.

Dolga AM, N. I., Ostroveanu A, Ten Bosch Q, Luiten PG, Eisel UL. (2008). "Lovastatin induces neuroprotection through tumor necrosis factor receptor 2 signaling pathways." J Alzheimers Dis **13**(2): 111-122.

Dong, Y., Yang, L., Zhao, H., Zhang, C. and Wu, D. (2014). "Transplantation of neurotrophin-3-transfected bone marrow mesenchymal stem cells for the repair of spinal cord injury." Neural Regen Res **9**(16): 1520-1524.

Dvoriantchikova, G., Pappas, S., Luo, X., Ribeiro, M., Danek, D., Pelaez, D., Park, K. K. and Ivanov, D. (2016). "Virally delivered, constitutively active NFkappaB improves survival of injured retinal ganglion cells." Eur J Neurosci **44**(11): 2935-2943.

Eliwa, H., Belzung, C. and Surget, A. (2017). "Adult hippocampal neurogenesis: Is it the alpha and omega of antidepressant action?" Biochem Pharmacol **141**: 86-99.

Elmore, S. (2007). "Apoptosis: a review of programmed cell death." Toxicol Pathol **35**(4): 495-516.

Emgard, M., Piao, J., Aineskog, H., Liu, J., Calzarossa, C., Odeberg, J., Holmberg, L., Samuelsson, E. B., Bezubik, B., Vincent, P. H., Falci, S. P., Seiger, A., Akesson, E. and Sundstrom, E. (2014). "Neuroprotective effects of human spinal cord-derived neural precursor cells after transplantation to the injured spinal cord." Experimental Neurology **253**: 138-145.

Eriksson, P. S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A. M., Nordborg, C., Peterson, D. A. and Gage, F. H. (1998). "Neurogenesis in the adult human hippocampus." Nat Med **4**(11): 1313-1317.

Ernst, A., Alkass, K., Bernard, S., Salehpour, M., Perl, S., Tisdale, J., Possnert, G., Druid, H. and Frisen, J. (2014). "Neurogenesis in the striatum of the adult human brain." Cell **156**(5): 1072-1083.

Erturk, A., Hellal, F., Enes, J. and Bradke, F. (2007). "Disorganized microtubules underlie the formation of retraction bulbs and the failure of axonal regeneration." J Neurosci **27**(34): 9169-9180.

Fan, C., Li, X., Xiao, Z., Zhao, Y., Liang, H., Wang, B., Han, S., Xu, B., Wang, N., Liu, S., Xue, W. and Dai, J. (2017). "A modified collagen scaffold facilitates endogenous neurogenesis for acute spinal cord injury repair." Acta Biomater **51**: 304-316.

Fan, X., Wang, J. Z., Lin, X. M. and Zhang, L. (2017). "Stem cell transplantation for spinal cord injury: a meta-analysis of treatment effectiveness and safety." Neural Regen Res **12**(5): 815-825.

Fehlings, M. G., Tetreault, L. A., Wilson, J. R., Aarabi, B., Anderson, P., Arnold, P. M., Brodke, D. S., Burns, A. S., Chiba, K., Dettori, J. R., Furlan, J. C., Hawryluk, G., Holly, L. T., Howley, S., Jeji, T., Kalsi-Ryan, S., Kotter, M., Kurpad, S., Marino, R. J., Martin, A. R., Massicotte, E., Merli, G., Middleton, J. W., Nakashima, H., Nagoshi, N., Palmieri, K., Singh, A., Skelly, A. C., Tsai, E. C., Vaccaro, A., Yee, A. and Harrop, J. S. (2017). "A Clinical Practice Guideline for the Management of Patients With Acute Spinal Cord Injury and Central Cord Syndrome: Recommendations on the Timing (</=24 Hours Versus >24 Hours) of Decompressive Surgery." Global Spine J **7**(3 Suppl): 195S-202S.

Filous, A. R. and Schwab, J. M. (2018). "Determinants of Axon Growth, Plasticity, and Regeneration in the Context of Spinal Cord Injury." Am J Pathol **188**(1): 53-62.

Filous, A. R., Tran, A., Howell, C. J., Busch, S. A., Evans, T. A., Stallcup, W. B., Kang, S. H., Bergles, D. E., Lee, S. I., Levine, J. M. and Silver, J. (2014). "Entrapment via synaptic-like connections between NG2 proteoglycan+ cells and dystrophic

axons in the lesion plays a role in regeneration failure after spinal cord injury." J Neurosci **34**(49): 16369-16384.

Fonseca, A. F. B., Scheffer, J. P., Giraldi-Guimaraes, A., Coelho, B. P., Medina, R. M. and Oliveira, A. L. A. (2017). "Comparison among bone marrow mesenchymal stem and mononuclear cells to promote functional recovery after spinal cord injury in rabbits." Acta Cir Bras **32**(12): 1026-1035.

Francos-Quijorna, I., Amo-Aparicio, J., Martinez-Muriana, A. and Lopez-Vales, R. (2016). "IL-4 drives microglia and macrophages toward a phenotype conducive for tissue repair and functional recovery after spinal cord injury." Glia **64**(12): 2079-2092.

Fridmacher, V., Kaltschmidt, B., Goudeau, B., Ndiaye, D., Rossi, F. M., Pfeiffer, J., Kaltschmidt, C., Israel, A. and Memet, S. (2003). "Forebrain-specific neuronal inhibition of nuclear factor-kappaB activity leads to loss of neuroprotection." J Neurosci **23**(28): 9403-9408.

Fu, H. D., Wang, H. R. and Li, D. H. (2017). "BMP-7 accelerates the differentiation of rabbit mesenchymal stem cells into cartilage through the Wnt/beta-catenin pathway." Exp Ther Med **14**(6): 5424-5428.

Furst, R. and Zundorf, I. (2014). "Plant-derived anti-inflammatory compounds: hopes and disappointments regarding the translation of preclinical knowledge into clinical progress." Mediators Inflamm **2014**: 146832.

Garcia, E., Aguilar-Cevallos, J., Silva-Garcia, R. and Ibarra, A. (2016). "Cytokine and Growth Factor Activation In Vivo and In Vitro after Spinal Cord Injury." Mediators Inflamm **2016**: 9476020.

Gashmardi N1, Hosseini SE2, Mehrabani D3, Edalatmanesh MA2, Khodabandeh Z3. (2017). "Impacts of Bone Marrow Stem Cells on Caspase-3 Levels after Spinal Cord Injury in Mice." Iran J Med Sci.: 593-598.

Gensel, J. C., Wang, Y., Guan, Z., Beckwith, K. A., Braun, K. J., Wei, P., McTigue, D. M. and Popovich, P. G. (2015). "Toll-Like Receptors and Dectin-1, a C-Type Lectin Receptor, Trigger Divergent Functions in CNS Macrophages." J Neurosci **35**(27): 9966-9976.

Gokce, E. C., Kahveci, R., Gokce, A., Sargon, M. F., Kisa, U., Aksoy, N., Cemil, B. and Erdogan, B. (2016). "Curcumin Attenuates Inflammation, Oxidative Stress, and Ultrastructural Damage Induced by Spinal Cord Ischemia-Reperfusion Injury in Rats." J Stroke Cerebrovasc Dis **25**(5): 1196-1207.

Goldstein, B., Little, J. W. and Harris, R. M. (1997). "Axonal sprouting following incomplete spinal cord injury: an experimental model." J Spinal Cord Med **20**(2): 200-206.

Gonzalez, R., Moffatt, G., Hagner, A., Sinha, S., Shin, W., Rahmani, W., Chojnacki, A. and Biernaskie, J. (2017). "Platelet-derived growth factor signaling modulates adult hair follicle dermal stem cell maintenance and self-renewal." NPJ Regen Med **2**: 11.

Gray, C. M., Remouchamps, C., McCorkell, K. A., Solt, L. A., Dejardin, E., Orange, J. S. and May, M. J. (2014). "Noncanonical NF-kappaB signaling is limited by classical NF-kappaB activity." Sci Signal **7**(311): ra13.

Gundimeda, U., McNeill, T. H., Barseghian, B. A., Tzeng, W. S., Rayudu, D. V., Cadenas, E. and Gopalakrishna, R. (2015). "Polyphenols from green tea prevent antineuritogenic action of Nogo-A via 67-kDa laminin receptor and hydrogen peroxide." Journal of Neurochemistry **132**(1): 70-84.

Gupta, D., Grant, D. M., Zakir Hossain, K. M., Ahmed, I. and Sottile, V. (2017). "Role of geometrical cues in bone marrow-derived mesenchymal stem cell survival, growth and osteogenic differentiation." J Biomater Appl: 885328217745699.

Hagglund, M. G., Hellsten, S. V., Bagchi, S., Philippot, G., Lofqvist, E., Nilsson, V. C., Almkvist, I., Karlsson, E., Sreedharan, S., Tafreshiha, A. and Fredriksson, R. (2015). "Transport of L-glutamine, L-alanine, L-arginine and L-histidine by the neuron-specific Slc38a8 (SNAT8) in CNS." J Mol Biol **427**(6 Pt B): 1495-1512.

Hall, E. D., Wang, J. A., Bosken, J. M. and Singh, I. N. (2016). "Lipid peroxidation in brain or spinal cord mitochondria after injury." J Bioenerg Biomembr **48**(2): 169-174.

Han, D., Wu, C., Xiong, Q., Zhou, L. and Tian, Y. (2015). "Anti-inflammatory Mechanism of Bone Marrow Mesenchymal Stem Cell Transplantation in Rat Model of Spinal Cord Injury." Cell Biochem Biophys **71**(3): 1341-1347.

Hawryluk, G. W., Spano, S., Chew, D., Wang, S., Erwin, M., Chamankhah, M., Forgione, N. and Fehlings, M. G. (2014). "An examination of the mechanisms by which neural precursors augment recovery following spinal cord injury: a key role for remyelination." Cell Transplant **23**(3): 365-380.

Hertz, L. and Rothman, D. L. (2017). "Glutamine-Glutamate Cycle Flux Is Similar in Cultured Astrocytes and Brain and Both Glutamate Production and Oxidation Are Mainly Catalyzed by Aspartate Aminotransferase." Biology (Basel) **6**(1).

Hong, J. Y., Lee, S. H., Lee, S. C., Kim, J. W., Kim, K. P., Kim, S. M., Tapia, N., Lim, K. T., Kim, J., Ahn, H. S., Ko, K., Shin, C. Y., Lee, H. T., Scholer, H. R., Hyun, J. K. and Han, D. W. (2014). "Therapeutic potential of induced neural stem cells for spinal cord injury." J Biol Chem **289**(47): 32512-32525.

Hosseini, S. M., Sani, M., Haider, K. H., Dorvash, M., Ziaee, S. M., Karimi, A. and Namavar, M. R. (2018). "Concomitant use of mesenchymal stem cells and neural stem cells for treatment of spinal cord injury: A combo cell therapy approach." Neurosci Lett **668**: 138-146.

Hu, J., Yang, Z., Li, X. and Lu, H. (2016). "C-C motif chemokine ligand 20 regulates neuroinflammation following spinal cord injury via Th17 cell recruitment." J Neuroinflammation **13**(1): 162.

Hu, J. G., Shi, L. L., Chen, Y. J., Xie, X. M., Zhang, N., Zhu, A. Y., Jiang, Z. S., Feng, Y. F., Zhang, C., Xi, J. and Lu, H. Z. (2016). "Differential effects of myelin basic protein-activated Th1 and Th2 cells on the local immune microenvironment of injured spinal cord." Experimental Neurology **277**: 190-201.

Hwang, D. H., Shin, H. Y., Kwon, M. J., Choi, J. Y., Ryu, B. Y. and Kim, B. G. (2014). "Survival of neural stem cell grafts in the lesioned spinal cord is enhanced by a combination of treadmill locomotor training via insulin-like growth factor-1 signaling." J Neurosci **34**(38): 12788-12800.

Chang, C. H., Chen, H. X., Yu, G., Peng, C. C. and Peng, R. Y. (2014). "Curcumin-Protected PC12 Cells Against Glutamate-Induced Oxidative Toxicity." Food Technol Biotechnol **52**(4): 468-478.

Chen, J., Zhang, Z., Liu, J., Zhou, R., Zheng, X., Chen, T., Wang, L., Huang, M., Yang, C., Li, Z., Bai, X. and Jin, D. (2014). "Acellular spinal cord scaffold seeded with bone marrow stromal cells protects tissue and promotes functional recovery in spinal cord-injured rats." J Neurosci Res **92**(3): 307-317.

Chen, S., Dong, Z., Cheng, M., Zhao, Y., Wang, M., Sai, N., Wang, X., Liu, H., Huang, G. and Zhang, X. (2017). "Homocysteine exaggerates microglia activation and

neuroinflammation through microglia localized STAT3 overactivation following ischemic stroke." J Neuroinflammation **14**(1): 187.

Chen, Y. J., Zhu, H., Zhang, N., Shen, L., Wang, R., Zhou, J. S., Hu, J. G. and Lu, H. Z. (2015). "Temporal kinetics of macrophage polarization in the injured rat spinal cord." J Neurosci Res **93**(10): 1526-1533.

Cheng, I., Park, D. Y., Mayle, R. E., Githens, M., Smith, R. L., Park, H. Y., Hu, S. S., Alamin, T. F., Wood, K. B. and Kharazi, A. I. (2017). "Does timing of transplantation of neural stem cells following spinal cord injury affect outcomes in an animal model?" J Spine Surg **3**(4): 567-571.

Cheng, Z., Zhu, W., Cao, K., Wu, F., Li, J., Wang, G., Li, H., Lu, M., Ren, Y. and He, X. (2016). "Anti-Inflammatory Mechanism of Neural Stem Cell Transplantation in Spinal Cord Injury." Int J Mol Sci **17**(9).

Christian, F., Smith, E. L. and Carmody, R. J. (2016). "The Regulation of NF-kappaB Subunits by Phosphorylation." Cells **5**(1).

Ihunwo, A. O., Tembo, L. H. and Dzamalala, C. (2016). "The dynamics of adult neurogenesis in human hippocampus." Neural Regen Res **11**(12): 1869-1883.

Ishige, K., Tanaka, M., Arakawa, M., Saito, H. and Ito, Y. (2005). "Distinct nuclear factor-kappaB/Rel proteins have opposing modulatory effects in glutamate-induced cell death in HT22 cells." Neurochem Int **47**(8): 545-555.

Ji, X. C., Dang, Y. Y., Gao, H. Y., Wang, Z. T., Gao, M., Yang, Y., Zhang, H. T. and Xu, R. X. (2015). "Local Injection of Lenti-BDNF at the Lesion Site Promotes M2 Macrophage Polarization and Inhibits Inflammatory Response After Spinal Cord Injury in Mice." Cell Mol Neurobiol **35**(6): 881-890.

Jin, Y., Sura, K. and Fischer, I. (2012). "Differential effects of distinct central nervous system regions on cell migration and axonal extension of neural precursor transplants." J Neurosci Res **90**(11): 2065-2073.

Julio-Pieper, M., Flor, P. J., Dinan, T. G. and Cryan, J. F. (2011). "Exciting Times beyond the Brain: Metabotropic Glutamate Receptors in Peripheral and Non-Neural Tissues." Pharmacological Reviews **63**(1): 35-58.

Kaltschmidt, C., Kaltschmidt, B., Neumann, H., Wekerle, H. and Baeuerle, P. A. (1994). "Constitutive NF-kappa B activity in neurons." Mol Cell Biol **14**(6): 3981-3992.

Kanamori, K. and Ross, B. D. (2006). "Kinetics of glial glutamine efflux and the mechanism of neuronal uptake studied in vivo in mildly hyperammonemic rat brain." Journal of Neurochemistry **99**(4): 1103-1113.

Kerschensteiner, M., Schwab, M. E., Lichtman, J. W. and Misgeld, T. (2005). "In vivo imaging of axonal degeneration and regeneration in the injured spinal cord." Nat Med **11**(5): 572-577.

Kettenmann, H., Hanisch, U. K., Noda, M. and Verkhratsky, A. (2011). "Physiology of microglia." Physiol Rev **91**(2): 461-553.

Khalatbary, A. R. (2014). "Natural polyphenols and spinal cord injury." Iran Biomed J.: 120-129.

Khalatbary, A. R. and Ahmadvand, H. (2011). "Anti-inflammatory effect of the epigallocatechin gallate following spinal cord trauma in rat." Iran Biomed J.: 31-37.

Kierdorf, K. and Prinz, M. (2013). "Factors regulating microglia activation." Frontiers in Cellular Neuroscience **7**.

Kim, K. M., Heo, D. R., Lee, J. Y., Seo, C. S. and Chung, S. K. (2017). "High-efficiency generation of induced pluripotent stem cells from human foreskin

fibroblast cells using the Sagunja-tang herbal formula." BMC Complement Altern Med **17**(1): 529.

Kirkley, K. S., Popichak, K. A., Afzali, M. F., Legare, M. E. and Tjalkens, R. B. (2017). "Microglia amplify inflammatory activation of astrocytes in manganese neurotoxicity." Journal of Neuroinflammation **14**.

Klein, C. and Fishell, G. (2004). "Neural stem cells: progenitors or panacea?" Dev Neurosci **26**(2-4): 82-92.

Kobayashi, Y., Okada, Y., Itakura, G., Iwai, H., Nishimura, S., Yasuda, A., Nori, S., Hikishima, K., Konomi, T., Fujiyoshi, K., Tsuji, O., Toyama, Y., Yamanaka, S., Nakamura, M. and Okano, H. (2012). "Pre-evaluated safe human iPSC-derived neural stem cells promote functional recovery after spinal cord injury in common marmoset without tumorigenicity." PLoS One **7**(12): e52787.

Kong, X. and Gao, J. (2017). "Macrophage polarization: a key event in the secondary phase of acute spinal cord injury." J Cell Mol Med **21**(5): 941-954.

Kuang, X., Huang, Y., Gu, H. F., Zu, X. Y., Zou, W. Y., Song, Z. B. and Guo, Q. L. (2012). "Effects of intrathecal epigallocatechin gallate, an inhibitor of Toll-like receptor 4, on chronic neuropathic pain in rats." Eur J Pharmacol **676**(1-3): 51-56.

Lai, B. Q., Che, M. T., Du, B. L., Zeng, X., Ma, Y. H., Feng, B., Qiu, X. C., Zhang, K., Liu, S., Shen, H. Y., Wu, J. L., Ling, E. A. and Zeng, Y. S. (2016). "Transplantation of tissue engineering neural network and formation of neuronal relay into the transected rat spinal cord." Biomaterials **109**: 40-54.

Lang, H., Schulte, B. A., Zhou, D., Smythe, N., Spicer, S. S. and Schmiedt, R. A. (2006). "Nuclear factor kappaB deficiency is associated with auditory nerve degeneration and increased noise-induced hearing loss." J Neurosci **26**(13): 3541-3550.

Lawrence, T. (2009). "The Nuclear Factor NF-kB Pathway in Inflammation." Cold Spring Harb Perspect Biol. **1**(6).

Li, C., Chen, X., Qiao, S., Liu, X., Liu, C., Zhu, D., Su, J. and Wang, Z. (2014). "Melatonin lowers edema after spinal cord injury." Neural Regen Res **9**(24): 2205-2210.

Liang, P., Liu, J., Xiong, J., Liu, Q., Zhao, J., Liang, H., Zhao, L. and Tang, H. (2014). "Neural stem cell-conditioned medium protects neurons and promotes propriospinal neurons relay neural circuit reconnection after spinal cord injury." Cell Transplant **23 Suppl 1**: S45-56.

Liddelow, S. A., Guttenplan, K. A., Clarke, L. E., Bennett, F. C., Bohlen, C. J., Schirmer, L., Bennett, M. L., Munch, A. E., Chung, W. S., Peterson, T. C., Wilton, D. K., Frouin, A., Napier, B. A., Panicker, N., Kumar, M., Buckwalter, M. S., Rowitch, D. H., Dawson, V. L., Dawson, T. M., Stevens, B. and Barres, B. A. (2017). "Neurotoxic reactive astrocytes are induced by activated microglia." Nature **541**(7638): 481-487.

Lima, R., Monteiro, S., Lopes, J. P., Barradas, P., Vasconcelos, N. L., Gomes, E. D., Assuncao-Silva, R. C., Teixeira, F. G., Morais, M., Sousa, N., Salgado, A. J. and Silva, N. A. (2017). "Systemic Interleukin-4 Administration after Spinal Cord Injury Modulates Inflammation and Promotes Neuroprotection." Pharmaceuticals (Basel) **10**(4).

Lin, J., Huo, X. and Liu, X. (2017). ""mTOR Signaling Pathway": A Potential Target of Curcumin in the Treatment of Spinal Cord Injury." Biomed Res Int **2017**: 1634801.

Lin, W., Li, M., Li, Y., Sun, X., Li, X., Yang, F., Huang, Y. and Wang, X. (2014). "Bone marrow stromal cells promote neurite outgrowth of spinal motor neurons by means of neurotrophic factors in vitro." Neurol Sci **35**(3): 449-457.

Liu, S., Schackel, T., Weidner, N. and Puttagunta, R. (2017). "Biomaterial-Supported Cell Transplantation Treatments for Spinal Cord Injury: Challenges and Perspectives." Frontiers in Cellular Neuroscience **11**: 430.

Liu, W. G., Wang, Z. Y. and Huang, Z. S. (2011). "Bone marrow-derived mesenchymal stem cells expressing the bFGF transgene promote axon regeneration and functional recovery after spinal cord injury in rats." Neurol Res **33**(7): 686-693.

Lodi, A., Saha, A., Lu, X., Wang, B., Sentandreu, E., Collins, M., Kolonin, M. G., DiGiovanni, J. and Tiziani, S. (2017). "Combinatorial treatment with natural compounds in prostate cancer inhibits prostate tumor growth and leads to key modulations of cancer cell metabolism." NPJ Precis Oncol **1**.

Lu, P., Ceto, S., Wang, Y., Graham, L., Wu, D., Kumamaru, H., Staufenberg, E. and Tuszynski, M. H. (2017). "Prolonged human neural stem cell maturation supports recovery in injured rodent CNS." J Clin Invest **127**(9): 3287-3299.

Lu, P., Wang, Y., Graham, L., McHale, K., Gao, M., Wu, D., Brock, J., Blesch, A., Rosenzweig, E. S., Havton, L. A., Zheng, B., Conner, J. M., Marsala, M. and Tuszynski, M. H. (2012). "Long-distance growth and connectivity of neural stem cells after severe spinal cord injury." Cell **150**(6): 1264-1273.

Lu, P., Woodruff, G., Wang, Y., Graham, L., Hunt, M., Wu, D., Boehle, E., Ahmad, R., Poplawski, G., Brock, J., Goldstein, L. S. and Tuszynski, M. H. (2014). "Long-distance axonal growth from human induced pluripotent stem cells after spinal cord injury." Neuron **83**(4): 789-796.

Ma, S. F., Chen, Y. J., Zhang, J. X., Shen, L., Wang, R., Zhou, J. S., Hu, J. G. and Lu, H. Z. (2015). "Adoptive transfer of M2 macrophages promotes locomotor recovery in adult rats after spinal cord injury." Brain Behav Immun **45**: 157-170.

Maier, J., Kincaid, C., Pagenstecher, A. and Campbell, I. L. (2002). "Regulation of Signal Transducer and Activator of Transcription and Suppressor of Cytokine-Signaling Gene Expression in the Brain of Mice with Astrocyte-Targeted Production of Interleukin-12 or Experimental Autoimmune Encephalomyelitis." Am J Pathol **160**(1): 271-288.

Marcol, W., Slusarczyk, W., Larysz-Brysz, M., Labuzek, K., Kapustka, B., Staszkiewicz, R., Rosicka, P., Kalita, K., Weglarz, W. and Lewin-Kowalik, J. (2017). "Extended magnetic resonance imaging studies on the effect of classically activated microglia transplantation on white matter regeneration following spinal cord focal injury in adult rats." Exp Ther Med **14**(5): 4869-4877.

Marchetti, L., Klein, M., Schlett, K., Pfizenmaier, K. and Eisel, U. L. (2004). "Tumor necrosis factor (TNF)-mediated neuroprotection against glutamate-induced excitotoxicity is enhanced by N-methyl-D-aspartate receptor activation. Essential role of a TNF receptor 2-mediated phosphatidylinositol 3-kinase-dependent NF-kappa B pathway." J Biol Chem **279**(31): 32869-32881.

Marote, A., Pomeshchik, Y., Goldwurm, S., Collin, A., Lamas, N. J., Pinto, L., Salgado, A. J. and Roybon, L. (2018). "Generation of an induced pluripotent stem cell line (CSC-44) from a Parkinson's disease patient carrying a compound heterozygous mutation (c.823C>T and EX6 del) in the PARK2 gene." Stem Cell Res **27**: 90-94.

Martin, W. (2009). "Nitroxyl anion--the universal signalling partner of endogenously produced nitric oxide?" Br J Pharmacol **157**(4): 537-539.

Mattson, M. P. and Meffert, M. K. (2006). "Roles for NF-kappaB in nerve cell survival, plasticity, and disease." Cell Death Differ **13**(5): 852-860.

McKenna, M. C., Stridh, M. H., McNair, L. F., Sonnewald, U., Waagepetersen, H. S. and Schousboe, A. (2016). "Glutamate oxidation in astrocytes: Roles of glutamate dehydrogenase and aminotransferases." J Neurosci Res **94**(12): 1561-1571.

Min-Jean Yin, L. B. C., Yumi Yamamoto, Youn-Tae Kwak, Shuichan Xu, Frank Mercurio, Miguel Barbosa, Melanie H. Cobb, Richard B. Gaynor (1988). "HTLV-I Tax Protein Binds to MEKK1 to Stimulate IκB Kinase Activity and NF-κB Activation." Cell **93**(5): 875–884.

Mireille Delhase, M. H., Yi Chen, Michael Karin (1999). "Positive and Negative Regulation of IκB Kinase Activity Through IKKβ Subunit Phosphorylation." Science **284**(5412): 309-313.

Nemati, S., Jabbari, R., Hajinasrollah, M., Mehrjerdi, N. Z., Azizi, H., Hemmesi, K., Moghiminasr, R., Azhdari, Z., Talebi, A., Mohitmafi, S., Dizaj, A. V. T., Sharifi, G., Baharvand, H., Rezaee, O. and Kiani, S. (2014). "Transplantation of Adult Monkey Neural Stem Cells into A Contusion Spinal Cord Injury Model in Rhesus Macaque Monkeys." Cell Journal **16**(2): 117-130.

Nori, S., Okada, Y., Yasuda, A., Tsuji, O., Takahashi, Y., Kobayashi, Y., Fujiyoshi, K., Koike, M., Uchiyama, Y., Ikeda, E., Toyama, Y., Yamanaka, S., Nakamura, M. and Okano, H. (2011). "Grafted human-induced pluripotent stem-cell-derived neurospheres promote motor functional recovery after spinal cord injury in mice." Proc Natl Acad Sci U S A **108**(40): 16825-16830.

Nutt, S. E., Chang, E. A., Suhr, S. T., Schlosser, L. O., Mondello, S. E., Moritz, C. T., Cibelli, J. B. and Horner, P. J. (2013). "Caudalized human iPSC-derived neural progenitor cells produce neurons and glia but fail to restore function in an early chronic spinal cord injury model." Experimental Neurology **248**: 491-503.

Oh, J., Lee, K. I., Kim, H. T., You, Y., Yoon, D. H., Song, K. Y., Cheong, E., Ha, Y. and Hwang, D. Y. (2015). "Human-induced pluripotent stem cells generated from intervertebral disc cells improve neurologic functions in spinal cord injury." Stem Cell Res Ther **6**: 125.

Oh, S. K., Choi, K. H., Yoo, J. Y., Kim, D. Y., Kim, S. J. and Jeon, S. R. (2016). "A Phase III Clinical Trial Showing Limited Efficacy of Autologous Mesenchymal Stem Cell Therapy for Spinal Cord Injury." Neurosurgery **78**(3): 436-447; discussion 447.

Oh, S. K. and Jeon, S. R. (2016). "Current Concept of Stem Cell Therapy for Spinal Cord Injury: A Review." Korean J Neurotrauma **12**(2): 40-46.

Orrenius, S., Zhivotovsky, B. and Nicotera, P. (2003). "Regulation of cell death: the calcium-apoptosis link." Nat Rev Mol Cell Biol **4**(7): 552-565.

Pal, R., Gopinath, C., Rao, N. M., Banerjee, P., Krishnamoorthy, V., Venkataramana, N. K. and Totey, S. (2010). "Functional recovery after transplantation of bone marrow-derived human mesenchymal stromal cells in a rat model of spinal cord injury." Cytotherapy **12**(6): 792-806.

Pan, J. Z., Ni, L., Sodhi, A., Aguanno, A., Young, W. and Hart, R. P. (2002). "Cytokine activity contributes to induction of inflammatory cytokine mRNAs in spinal cord following contusion." J Neurosci Res **68**(3): 315-322.

Panatier, A. and Robitaille, R. (2016). "Astrocytic mGluR5 and the tripartite synapse." Neuroscience **323**: 29-34.

Park, K. W., Lee, D. Y., Joe, E. H., Kim, S. U. and Jin, B. K. (2005). "Neuroprotective role of microglia expressing interleukin-4." J Neurosci Res **81**(3): 397-402.

Pekny, M., Wilhelmsson, U. and Pekna, M. (2014). "The dual role of astrocyte activation and reactive gliosis." Neurosci Lett **565**: 30-38.

Penha, E. M., Meira, C. S., Guimaraes, E. T., Mendonca, M. V., Gravely, F. A., Pinheiro, C. M., Pinheiro, T. M., Barrouin-Melo, S. M., Ribeiro-Dos-Santos, R. and Soares, M. B. (2014). "Use of autologous mesenchymal stem cells derived from bone marrow for the treatment of naturally injured spinal cord in dogs." Stem Cells Int **2014**: 437521.

Pervin, M., Unno, K., Nakagawa, A., Takahashi, Y., Iguchi, K., Yamamoto, H., Hoshino, M., Hara, A., Takagaki, A., Nanjo, F., Minami, A., Imai, S. and Nakamura, Y. (2017). "Blood brain barrier permeability of (-)-epigallocatechin gallate, its proliferation-enhancing activity of human neuroblastoma SH-SY5Y cells, and its preventive effect on age-related cognitive dysfunction in mice." Biochem Biophys Rep **9**: 180-186.

Phillis JW, R. J., O'Regan MH. (2000). "Transporter reversal as a mechanism of glutamate release from the ischemic rat cerebral cortex: studies with DL-threo-beta-benzyloxyaspartate." Brain Res. **880**(1-2): 105–112.

Pizzi, M., Goffi, F., Boroni, F., Benarese, M., Perkins, S. E., Liou, H. C. and Spano, P. (2002). "Opposing roles for NF-kappa B/Rel factors p65 and c-Rel in the modulation of neuron survival elicited by glutamate and interleukin-1beta." J Biol Chem **277**(23): 20717-20723.

Pizzi, M., Sarnico, I., Lanzillotta, A., Battistin, L. and Spano, P. (2009). "Post-ischemic brain damage: NF-kappaB dimer heterogeneity as a molecular determinant of neuron vulnerability." FEBS J **276**(1): 27-35.

Plemel, J. R., Chojnacki, A., Sparling, J. S., Liu, J., Plunet, W., Duncan, G. J., Park, S. E., Weiss, S. and Tetzlaff, W. (2011). "Platelet-derived growth factor-responsive neural precursors give rise to myelinating oligodendrocytes after transplantation into the spinal cords of contused rats and dysmyelinated mice." Glia **59**(12): 1891-1910.

Polentes, J., Jendelova, P., Cailleret, M., Braun, H., Romanyuk, N., Tropel, P., Brenot, M., Itier, V., Seminatore, C., Baldauf, K., Turnovcova, K., Jirak, D., Teletin, M., Come, J., Tournois, J., Reymann, K., Sykova, E., Viville, S. and Onteniente, B. (2012). "Human induced pluripotent stem cells improve stroke outcome and reduce secondary degeneration in the recipient brain." Cell Transplant **21**(12): 2587-2602.

Pollock, K., Stroemer, P., Patel, S., Stevanato, L., Hope, A., Miljan, E., Dong, Z., Hodges, H., Price, J. and Sinden, J. D. (2006). "A conditionally immortal clonal. stem cell line from human cortical neuroepithelium for the treatment of ischemic stroke." Experimental Neurology **199**(1): 143-155.

Pomeshchik, Y., Puttonen, K. A., Kidin, I., Ruponen, M., Lehtonen, S., Malm, T., Akesson, E., Hovatta, O. and Koistinaho, J. (2015). "Transplanted Human Induced Pluripotent Stem Cell-Derived Neural Progenitor Cells Do Not Promote Functional Recovery of Pharmacologically Immunosuppressed Mice With Contusion Spinal Cord Injury." Cell Transplant **24**(9): 1799-1812.

Prajapati, S. and Gaynor, R. B. (2002). "Regulation of Ikappa B kinase (IKK)gamma /NEMO function by IKKbeta -mediated phosphorylation." J Biol Chem **277**(27): 24331-24339.

Qian, Z. M., He, X., Liang, T., Wu, K. C., Yan, Y. C., Lu, L. N., Yang, G., Luo, Q. Q., Yung, W. H. and Ke, Y. (2014). "Lipopolysaccharides upregulate hepcidin in neuron via microglia and the IL-6/STAT3 signaling pathway." Mol Neurobiol **50**(3): 811-820.

Qu, J. and Zhang, H. (2017). "Roles of Mesenchymal Stem Cells in Spinal Cord Injury." Stem Cells Int **2017**: 5251313.

Rao, S. N. and Pearse, D. D. (2016). "Regulating Axonal Responses to Injury: The Intersection between Signaling Pathways Involved in Axon Myelination and The Inhibition of Axon Regeneration." Front Mol Neurosci **9**: 33.

Renno, W. M., Al-Khaledi, G., Mousa, A., Karam, S. M., Abul, H. and Asfar, S. (2014). "(-)-Epigallocatechin-3-gallate (EGCG) modulates neurological function when intravenously infused in acute and, chronically injured spinal cord of adult rats." Neuropharmacology **77**: 100-119.

Renno, W. M., Al-Maghrebi, M., Rao, M. S. and Khraishah, H. (2015). "(-)-Epigallocatechin-3-gallate modulates spinal cord neuronal degeneration by enhancing growth-associated protein 43, B-cell lymphoma 2, and decreasing B-cell lymphoma 2-associated x protein expression after sciatic nerve crush injury." J Neurotrauma **32**(3): 170-184.

Ridder, D. A. and Schwaninger, M. (2009). "NF-kappaB signaling in cerebral ischemia." Neuroscience **158**(3): 995-1006.

Rohl, C., Lucius, R. and Sievers, J. (2007). "The effect of activated microglia on astrogliosis parameters in astrocyte cultures." Brain Res **1129**(1): 43-52.

Romanyuk, N., Amemori, T., Turnovcova, K., Prochazka, P., Onteniente, B., Sykova, E. and Jendelova, P. (2015). "Beneficial Effect of Human Induced Pluripotent Stem Cell-Derived Neural Precursors in Spinal Cord Injury Repair." Cell Transplant **24**(9): 1781-1797.

Rosado, I. R., Carvalho, P. H., Alves, E. G., Tagushi, T. M., Carvalho, J. L., Silva, J. F., Lavor, M. S., Oliveira, K. M., Serakides, R., Goes, A. M. and Melo, E. G. (2017). "Immunomodulatory and neuroprotective effect of cryopreserved allogeneic mesenchymal stem cells on spinal cord injury in rats." Genet Mol Res **16**(1).

Rose, E. M., Koo, J. C., Antflick, J. E., Ahmed, S. M., Angers, S. and Hampson, D. R. (2009). "Glutamate transporter coupling to Na,K-ATPase." J Neurosci **29**(25): 8143-8155.

Roussos, P., Katsel, P., Davis, K. L., Giakoumaki, S. G., Lencz, T., Malhotra, A. K., Siever, L. J., Bitsios, P. and Haroutunian, V. (2013). "Convergent findings for abnormalities of the NF-kappaB signaling pathway in schizophrenia." Neuropsychopharmacology **38**(3): 533-539.

Salem, N., Salem, M. Y., Elmaghrabi, M. M., Elawady, M. A., Sabry, D., Shamaa, A., Elkasapy, A. H., Ibrhim, N. and Elamir, A. (2017). "Does vitamin C have the ability to augment the therapeutic effect of bone marrow-derived mesenchymal stem cells on spinal cord injury?" Neural Regen Res **12**(12): 2050-2058.

Saler, M., Caliogna, L., Botta, L., Benazzo, F., Riva, F. and Gastaldi, G. (2017). "hASC and DFAT, Multipotent Stem Cells for Regenerative Medicine: A Comparison of Their Potential Differentiation In Vitro." Int J Mol Sci **18**(12).

Salewski, R. P., Mitchell, R. A., Li, L., Shen, C., Milekovskaia, M., Nagy, A. and Fehlings, M. G. (2015). "Transplantation of Induced Pluripotent Stem Cell-Derived Neural Stem Cells Mediate Functional Recovery Following Thoracic Spinal Cord Injury Through Remyelination of Axons." Stem Cells Transl Med **4**(7): 743-754.

Sanivarapu, R., Vallabhaneni, V. and Verma, V. (2016). "The Potential of Curcumin in Treatment of Spinal Cord Injury." Neurol Res Int **2016**: 9468193.

Sareen, D., Gowing, G., Sahabian, A., Staggenborg, K., Paradis, R., Avalos, P., Latter, J., Ornelas, L., Garcia, L. and Svendsen, C. N. (2014). "Human induced

pluripotent stem cells are a novel source of neural progenitor cells (iNPCs) that migrate and integrate in the rodent spinal cord." J Comp Neurol **522**(12): 2707-2728.

SC, S. (2010). "Non-canonical NF-κB signaling pathway." Cell Research: 71–85.

Shih, R. H., Wang, C. Y. and Yang, C. M. (2015). "NF-kappaB Signaling Pathways in Neurological Inflammation: A Mini Review." Front Mol Neurosci **8**: 77.

Shihabuddin, L. S., Horner, P. J., Ray, J. and Gage, F. H. (2000). "Adult spinal cord stem cells generate neurons after transplantation in the adult dentate gyrus." J Neurosci **20**(23): 8727-8735.

Singh, V. K., Saini, A., Kalsan, M., Kumar, N. and Chandra, R. (2016). "Describing the Stem Cell Potency: The Various Methods of Functional Assessment and In silico Diagnostics." Front Cell Dev Biol **4**: 134.

Spalding, K. L., Bergmann, O., Alkass, K., Bernard, S., Salehpour, M., Huttner, H. B., Bostrom, E., Westerlund, I., Vial, C., Buchholz, B. A., Possnert, G., Mash, D. C., Druid, H. and Frisen, J. (2013). "Dynamics of hippocampal neurogenesis in adult humans." Cell **153**(6): 1219-1227.

Stammers, A. T., Liu, J. and Kwon, B. K. (2012). "Expression of inflammatory cytokines following acute spinal cord injury in a rodent model." J Neurosci Res **90**(4): 782-790.

Stenudd, M., Sabelström, H. and Frisén, J. (2015). "Role of Endogenous Neural Stem Cells in Spinal Cord Injury and Repair." JAMA Neurology **72**(2): 235.

Stephenson, D., Yin, T., Smalstig, E. B., Hsu, M. A., Panetta, J., Little, S. and Clemens, J. (2000). "Transcription factor nuclear factor-kappa B is activated in neurons after focal cerebral ischemia." J Cereb Blood Flow Metab **20**(3): 592-603.

Sun, F. and He, Z. (2010). "Neuronal intrinsic barriers for axon regeneration in the adult CNS." Curr Opin Neurobiol **20**(4): 510-518.

Sun, L., Li, M., Ma, X., Feng, H., Song, J., Lv, C. and He, Y. (2017). "Inhibition of HMGB1 reduces rat spinal cord astrocytic swelling and AQP4 expression after oxygen-glucose deprivation and reoxygenation via TLR4 and NF-kappaB signaling in an IL-6-dependent manner." J Neuroinflammation **14**(1): 231.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K. and Yamanaka, S. (2007). "Induction of pluripotent stem cells from adult human fibroblasts by defined factors." Cell **131**(5): 861-872.

Takahashi, K. and Yamanaka, S. (2006). "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors." Cell **126**(4): 663-676.

Tang, D. L., Kang, R., Coyne, C. B., Zeh, H. J. and Lotze, M. T. (2012). "PAMPs and DAMPs: signal 0s that spur autophagy and immunity." Immunological Reviews **249**: 158-175.

Tang, H., Sha, H., Sun, H., Wu, X., Xie, L., Wang, P., Xu, C., Larsen, C., Zhang, H. L., Gong, Y., Mao, Y., Chen, X., Zhou, L., Feng, X. and Zhu, J. (2013). "Tracking induced pluripotent stem cells-derived neural stem cells in the central nervous system of rats and monkeys." Cell Reprogram **15**(5): 435-442.

Tian, D. S., Dong, Q., Pan, D. J., He, Y., Yu, Z. Y., Xie, M. J. and Wang, W. (2007). "Attenuation of astrogliosis by suppressing of microglial proliferation with the cell cycle inhibitor olomoucine in rat spinal cord injury model." Brain Res **1154**: 206-214.

Tian, W., Han, X. G., Liu, Y. J., Tang, G. Q., Liu, B., Wang, Y. Q., Xiao, B. and Xu, Y. F. (2013). "Intrathecal epigallocatechin gallate treatment improves functional

recovery after spinal cord injury by upregulating the expression of BDNF and GDNF." Neurochem Res **38**(4): 772-779.

Tjalkens, R. B., Popichak, K. A. and Kirkley, K. A. (2017). "Inflammatory Activation of Microglia and Astrocytes in Manganese Neurotoxicity." Adv Neurobiol **18**: 159-181.

Torres-Espin, A., Hernandez, J. and Navarro, X. (2013). "Gene expression changes in the injured spinal cord following transplantation of mesenchymal stem cells or olfactory ensheathing cells." PLoS One **8**(10): e76141.

Torres-Espin, A., Redondo-Castro, E., Hernandez, J. and Navarro, X. (2014). "Bone marrow mesenchymal stromal cells and olfactory ensheathing cells transplantation after spinal cord injury--a morphological and functional comparison in rats." Eur J Neurosci **39**(10): 1704-1717.

Torres-Espin, A., Redondo-Castro, E., Hernandez, J. and Navarro, X. (2015). "Immunosuppression of allogenic mesenchymal stem cells transplantation after spinal cord injury improves graft survival and beneficial outcomes." J Neurotrauma **32**(6): 367-380.

Tsuji, O., Miura, K., Fujiyoshi, K., Momoshima, S., Nakamura, M. and Okano, H. (2011). "Cell therapy for spinal cord injury by neural stem/progenitor cells derived from iPS/ES cells." Neurotherapeutics **8**(4): 668-676.

Vanicky, I., Urdzikova, L., Saganova, K., Cizkova, D. and Galik, J. (2001). "A simple and reproducible model of spinal cord injury induced by epidural balloon inflation in the rat." J Neurotrauma **18**(12): 1399-1407.

Venkatesh, I. and Blackmore, M. G. (2017). "Selecting optimal combinations of transcription factors to promote axon regeneration: Why mechanisms matter." Neurosci Lett **652**: 64-73.

Veremeyko, T., Yung, A. W. Y., Dukhinova, M., Kuznetsova, I. S., Pomytkin, I., Lyundup, A., Strekalova, T., Barteneva, N. S. and Ponomarev, E. D. (2018). "Cyclic AMP Pathway Suppress Autoimmune Neuroinflammation by Inhibiting Functions of Encephalitogenic CD4 T Cells and Enhancing M2 Macrophage Polarization at the Site of Inflammation." Front Immunol **9**: 50.

Wan, F. and Lenardo, M. J. (2009). "Specification of DNA binding activity of NF-kappaB proteins." Cold Spring Harb Perspect Biol **1**(4): a000067.

Wang, L. J., Zhang, R. P. and Li, J. D. (2014). "Transplantation of neurotrophin-3-expressing bone mesenchymal stem cells improves recovery in a rat model of spinal cord injury." Acta Neurochir (Wien) **156**(7): 1409-1418.

Wang, Q., Yu, S., Simonyi, A., Sun, G. Y. and Sun, A. Y. (2005). "Kainic Acid-Mediated Excitotoxicity as a Model for Neurodegeneration." Molecular Neurobiology **31**(1-3): 003-016.

Wang, X., Cao, K., Sun, X., Chen, Y., Duan, Z., Sun, L., Guo, L., Bai, P., Sun, D., Fan, J., He, X., Young, W. and Ren, Y. (2015). "Macrophages in spinal cord injury: phenotypic and functional change from exposure to myelin debris." Glia **63**(4): 635-651.

Wilcox, J. T., Satkunendrarajah, K., Zuccato, J. A., Nassiri, F. and Fehlings, M. G. (2014). "Neural precursor cell transplantation enhances functional recovery and reduces astrogliosis in bilateral compressive/contusive cervical spinal cord injury." Stem Cells Transl Med **3**(10): 1148-1159.

Wright, K. T., El Masri, W., Osman, A., Chowdhury, J. and Johnson, W. E. (2011). "Concise review: Bone marrow for the treatment of spinal cord injury: mechanisms and clinical applications." Stem Cells **29**(2): 169-178.

Wu, Y., Lin, Y. H., Shi, L. L., Yao, Z. F., Xie, X. M., Jiang, Z. S., Tang, J., Hu, J. G. and Lu, H. Z. (2017). "Temporal kinetics of CD8(+) CD28(+) and CD8(+) CD28(-) T lymphocytes in the injured rat spinal cord." J Neurosci Res **95**(8): 1666-1676.

Xiong, L. L., Liu, F., Lu, B. T., Zhao, W. L., Dong, X. J., Liu, J., Zhang, R. P., Zhang, P. and Wang, T. H. (2017). "Bone Marrow Mesenchymal Stem-Cell Transplantation Promotes Functional Improvement Associated with CNTF-STAT3 Activation after Hemi-Sectioned Spinal Cord Injury in Tree Shrews." Frontiers in Cellular Neuroscience **11**: 172.

Xu, B., Zhao, Y., Xiao, Z., Wang, B., Liang, H., Li, X., Fang, Y., Han, S., Fan, C. and Dai, J. (2017). "A Dual Functional Scaffold Tethered with EGFR Antibody Promotes Neural Stem Cell Retention and Neuronal Differentiation for Spinal Cord Injury Repair." Adv Healthc Mater **6**(9).

Yabe, T., Wilson, D. and Schwartz, J. P. (2001). "NFkappaB activation is required for the neuroprotective effects of pigment epithelium-derived factor (PEDF) on cerebellar granule neurons." J Biol Chem **276**(46): 43313-43319.

Yaguchi, M., Ohta, S., Toyama, Y., Kawakami, Y. and Toda, M. (2008). "Functional recovery after spinal cord injury in mice through activation of microglia and dendritic cells after IL-12 administration." J Neurosci Res **86**(9): 1972-1980.

Yan, J., Xu, L., Welsh, A. M., Hatfield, G., Hazel, T., Johe, K. and Koliatsos, V. E. (2007). "Extensive neuronal differentiation of human neural stem cell grafts in adult rat spinal cord." PLoS Med **4**(2): e39.

Yan, X., Huang, G., Liu, Q., Zheng, J., Chen, H., Huang, Q., Chen, J. and Huang, H. (2017). "Withaferin A protects against spinal cord injury by inhibiting apoptosis and inflammation in mice." Pharm Biol **55**(1): 1171-1176.

Yang, G. and Tang, W. Y. (2017). "Resistance of interleukin-6 to the extracellular inhibitory environment promotes axonal regeneration and functional recovery following spinal cord injury." Int J Mol Med **39**(2): 437-445.

Yang, M. S., Park, E. J., Sohn, S., Kwon, H. J., Shin, W. H., Pyo, H. K., Jin, B., Choi, K. S., Jou, I. and Joe, E. H. (2002). "Interleukin-13 and -4 induce death of activated microglia." Glia **38**(4): 273-280.

Yao, M., Yang, L., Wang, J., Sun, Y. L., Dun, R. L., Wang, Y. J. and Cui, X. J. (2015). "Neurological recovery and antioxidant effects of curcumin for spinal cord injury in the rat: a network meta-analysis and systematic review." J Neurotrauma **32**(6): 381-391.

Yaster, M., Guan, X. W., Petralia, R. S., Rothstein, J. D., Lu, W. and Tao, Y. X. (2011). "Effect of Inhibition of Spinal Cord Glutamate Transporters on Inflammatory Pain Induced by Formalin and Complete Freund's Adjuvant." Anesthesiology **114**(2): 412-423.

Yin, F., Guo, L., Meng, C. Y., Liu, Y. J., Lu, R. F., Li, P. and Zhou, Y. B. (2014). "Transplantation of mesenchymal stem cells exerts anti-apoptotic effects in adult rats after spinal cord ischemia-reperfusion injury." Brain Res **1561**: 1-10.

Yousefifard, M., Nasirinezhad, F., Shardi Manaheji, H., Janzadeh, A., Hosseini, M. and Keshavarz, M. (2016). "Human bone marrow-derived and umbilical cord-derived mesenchymal stem cells for alleviating neuropathic pain in a spinal cord injury model." Stem Cell Res Ther **7**: 36.

Yousefifard, M., Rahimi-Movaghar, V., Nasirinezhad, F., Baikpour, M., Safari, S., Saadat, S., Moghadas Jafari, A., Asady, H., Razavi Tousi, S. M. and Hosseini, M. (2016). "Neural stem/progenitor cell transplantation for spinal cord injury treatment; A systematic review and meta-analysis." Neuroscience **322**: 377-397.

Yu, J., Vodyanik, M. A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J. L., Tian, S., Nie, J., Jonsdottir, G. A., Ruotti, V., Stewart, R., Slukvin, II and Thomson, J. A. (2007). "Induced pluripotent stem cell lines derived from human somatic cells." Science **318**(5858): 1917-1920.

Yuan, J., Liu, W., Zhu, H., Chen, Y., Zhang, X., Li, L., Chu, W., Wen, Z., Feng, H. and Lin, J. (2017). "Curcumin inhibits glial scar formation by suppressing astrocyte-induced inflammation and fibrosis in vitro and in vivo." Brain Res **1655**: 90-103.

Yuste, J. E., Tarragon, E., Campuzano, C. M. and Ros-Bernal, F. (2015). "Implications of glial nitric oxide in neurodegenerative diseases." Frontiers in Cellular Neuroscience **9**.

Zabel, M., Nackenoff, A., Kirsch, W. M., Harrison, F., Perry, G. and Schrag, M. (2017). "Markers of oxidative damage to lipids, nucleic acids and proteins and antioxidant enzymes activities in Alzheimer's disease brain: a meta-analysis in human pathological specimens." Free Radic Biol Med.

Zeng, X., Zeng, Y. S., Ma, Y. H., Lu, L. Y., Du, B. L., Zhang, W., Li, Y. and Chan, W. Y. (2011). "Bone marrow mesenchymal stem cells in a three-dimensional gelatin sponge scaffold attenuate inflammation, promote angiogenesis, and reduce cavity formation in experimental spinal cord injury." Cell Transplant **20**(11-12): 1881-1899.

Zhang, N., Wei, G., Ye, J., Yang, L., Hong, Y., Liu, G., Zhong, H. and Cai, X. (2017). "Effect of curcumin on acute spinal cord injury in mice via inhibition of inflammation and TAK1 pathway." Pharmacol Rep **69**(5): 1001-1006.

Zhang, Y., Liu, J., Yao, S., Li, F., Xin, L., Lai, M., Bracchi-Ricard, V., Xu, H., Yen, W., Meng, W., Liu, S., Yang, L., Karmally, S., Zhu, H., Gordon, J., Khalili, K., Srinivasan, S., Bethea, J. R., Mo, X. and Hu, W. (2012). "Nuclear factor kappa B signaling initiates early differentiation of neural stem cells." Stem Cells **30**(3): 510-524.

Zhao, J., Wang, L. and Li, Y. (2017). "Electroacupuncture alleviates the inflammatory response via effects on M1 and M2 macrophages after spinal cord injury." Acupunct Med **35**(3): 224-230.

Zhu, Y., Uezono, N., Yasui, T. and Nakashima, K. (2018). "Neural stem cell therapy aiming at better functional recovery after spinal cord injury." Dev Dyn **247**(1): 75-84.

Zu, J., Wang, Y., Xu, G., Zhuang, J., Gong, H. and Yan, J. (2014). "Curcumin improves the recovery of motor function and reduces spinal cord edema in a rat acute spinal cord injury model by inhibiting the JAK/STAT signaling pathway." Acta Histochem **116**(8): 1331-1336.